

# Practical Physiological Chemistry.

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With an INTRODUCTION by

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TO THE  
PRESENT AND PAST MEMBERS  
AND THE  
SECRETARY  
OF THE  
MEDICAL RESEARCH COMMITTEE

this book is humbly

DEDICATED

*by the Author as a very slight token of gratitude for  
generous help received and in appreciation of  
the brilliant results already achieved  
in many fields due to  
their inception of  
new methods  
of attack.*



## INTRODUCTION.

My colleague's book, in its earlier editions, received so hearty and so general a welcome that any personal words of recommendation seem now uncalled for. In this edition, however, it emerges as a work of a somewhat different order.

Like all good books which deal with a progressive subject, it has felt the growth impulse, and, notwithstanding the exceptional nature of the times, material for growth has during the last few years accumulated in abundance. If this edition has largely outgrown its predecessors the increase is not only desirable, but, in my opinion, necessary.

Progress in any science calls continuously for new methods, and for extension and improvement in technique. In the growth of any branch of knowledge there are, indeed, periods when the development of technique becomes the most pressing of needs, and its success the best measure of progress. Biochemistry has been successfully passing through such a period. Its methods have been greatly multiplied, extended and improved. We are now beginning to reap the reward in the accumulation of accurate quantitative data. One need peculiar to biochemistry, that of following changes in living tissues without terminating the life of the animal, or harming the human subject, has now been largely met, at least so far as studies of the blood are concerned. This is due to the success of micro-methods of analysis. So significant are some of the results which can be obtained that we may hope to see the methods become as general in connexion with medical diagnosis as the use of the stethoscope or the electrocardiogram.

It may be good for the advanced student, possessed of leisure, to determine for himself the exact conditions necessary for success in the use of a given method. For two classes, however, it is highly desirable that success should be reached as immediately as possible: for the elementary student, in order that his faith may not be weakened, and for the research worker not specially versed in chemical technique, who, with limited time, wishes to apply a method to medical or biological problems. Each of these will be

the better for descriptions which secure against failure. Such are the descriptions found in this book. Indeed, the chief satisfaction I derive from being allowed to write this foreword arises from the opportunity it gives me of bearing witness to the fact that the author has always a first-hand acquaintance with his subject matter. In connexion with the newer, and less familiar, tests and methods the directions are not copied from elsewhere, not even (when they are due to others) from the descriptions of their originators. They have been written at the laboratory bench, step by step with the successful accomplishment of the process they describe. When success has seemed doubtful, or too difficult of attainment, the method has found no place in the book. Older methods have often been modified in detail, as a result of long experience of their use in practical classes. On the other hand not a few of the processes described are original. It is indeed a pleasant duty to emphasise the fact that the book is used as a medium for the publication of a considerable amount of patient research work requiring abilities of a special order. It is to be trusted that this work will receive the same degree of recognition that it undoubtedly would if it were published through the more ordinary channels of the scientific periodicals.

Some of the sections are intended for purposes wider than that of class instruction alone. In the chapter on the preparation of the amino-acids for instance, Mr. Cole has drawn on the collective experience of many workers in my laboratory. The descriptions are unique in their wealth of detail, and I feel confident that the preparation of these compounds by the methods described can be undertaken with every prospect of success by all workers. A supply of pure amino-acids is so important for the prosecution of many lines of research that the inclusion of the chapter will be welcomed by many who have been disappointed at the results of their previous attempts. Of my own knowledge I can testify to the success that has attended the preparation of histidine and tryptophane, for example, by junior laboratory attendants following the descriptions here published.

In my experience the teaching of practical biochemistry to students of physiology presents a difficulty less felt in the practical teaching of the other branches of the science. A successful histo-

logical preparation, or the neat accomplishment of a graphic record, yields immediate satisfaction to a student biologically inclined. He is dealing directly with the animal, and the result seems an end in itself. It is otherwise with tests and estimations carried out as mere exercises. The interest of a quantitative result, which may be great when it is obtained during an actual study of metabolism, seems remote to the student who works without any such stimulus. Yet in large chemical classes it is almost impossible to provide closer touch with the animal, and interest cannot always be secured by maintaining an exact correspondence in the sequence of lectures and classes. It is desirable therefore that, even in a book with aims that are avowedly practical, there should be some judicious reference to theory and to the actual significance of results. In the present work this end seems to be reached without undue consumption of space.

The book in its present form, while very fully covering the ground required by the medical student, can be profitably used by all who seek for accurate and full descriptions of biochemical methods, whether for use in medical diagnosis or in biological researches. The earlier editions of the book have been used by the students at the Agricultural Laboratory here, and in its present form it would appear to be highly suited to the needs of those engaged in the study of animal nutrition.

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## FROM THE PREFACE TO THE FIFTH EDITION

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I have added a considerable amount of new material, the most important being a chapter on the properties of solutions in which particular attention is paid to the hydrogen-ion concentration and to the colloidal state: a chapter on the preparation and properties of certain of the amino-acids: on the preparation and hydrolysis of nucleic acid: sections on the asymmetric carbon atom and on the theory of the polarimeter: on the action of intestinal bacteria on proteins: on autolysis: on the action of oxidase systems and many new quantitative methods related to enzyme action and blood, urinary and gastric analyses. A good deal of this material has not been published previously, but the methods have stood the test of routine class work at Cambridge, and it is trusted that they will not fail when tried elsewhere.

With the exception of the exercises on the preparation of the amino-acids and on the hydrolysis of nucleic acid, the book represents the course in Practical Physiological Chemistry for Medical Students at Cambridge. It may be objected that the course is overburdened with analytical exercises. They are inserted for two reasons that seem important to my Chief, Prof. F. G. Hopkins, and to myself. In the first place they have considerable educational value: one is enabled to train the student to make accurate observations and pay attention to the effect of variations in conditions on results. Secondly, it is hoped that the student, having acquired the technique necessary to determine the course of the metabolic changes in the normal individual, will be encouraged to extend his observations to those occurring in disease. Progress in medical science is largely dependent on the statistical method. I am convinced that a considerable body of trained medical men making accurate analyses of the abundant clinical material that must inevitably come their way will advance our knowledge much more rapidly than a few isolated specialists, who are apt to confine their attention to subjects in advanced disease. It is the border-land between health and ill-health that particularly requires exhaustive investigation, and for its exploration a whole army is required. We can safely rely on the Medical Research Committee for the Staff work necessary for the proper co-ordination of the results.

## PREFACE TO THE SIXTH EDITION.

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THE present edition has been carefully revised and several new methods introduced. The most important of these is my micro-method for the determination of blood sugar, which my Cambridge students have used for nearly a year with very consistent results. The method is a modification of McLean's method which in my hands and in class work proved most unsatisfactory. It is a substitute for Bang's micro-method, which, though reliable in the hands of the expert, has been found too difficult for the average student.

For the estimation of blood chlorides, Van Slyke's excellent method is described in preference to Bang's micro-method. The Soya bean method for the estimation of the urea of blood also finds a place, owing to its importance in the diagnosis of renal disease.

I am very grateful to my many correspondents for criticisms and suggestions, which enable me to render the book helpful to as wide a class of worker as possible.

Chemicals and apparatus of pre-war standard are still difficult to obtain, but Messrs. Baird and Tatlock, of London, are making a praiseworthy effort to maintain a stock of all the essential apparatus and reagents required for the various exercises described. I have to thank them for the loan of the blocks of several new figures.

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*October, 1920.*

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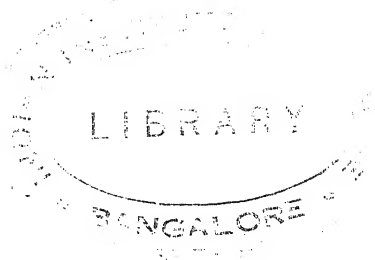
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## CHAPTER I.

### THE PROPERTIES OF SOLUTIONS.

#### A. Colloids and Crystalloids.

The condition of a substance in "solution" is one that differs considerably with different substances; moreover, it may differ with the same substance, depending on the method of preparing the solution. All "solutions" can be regarded as suspensions of particles in the "solvent." The size and nature of the particles cause variations in the physical properties of the solution.

There are four classes of so-called "solutions," which are not, however, very sharply differentiated from one another.

They are

CRYSTALLOIDS { *ELECTROLYTES.*  
*NON-ELECTROLYTES.*

COLLOIDS { *EMULSOIDS.*  
*SUSPENSIDS.*

Probably the essential difference between colloids and crystalloids is the size of the particles suspended in the fluid. In the crystalloids these particles are small, consisting of ions or single, relatively small molecules. In the colloids the particles are large, either because the molecules themselves are large, or because they tend to aggregate and form relatively large masses. The difference between emulsoids and suspensoids is probably that suspensoids are two-phase liquids in which the "solvent" (external phase) does not combine with the "solute" (internal phase), that is to say, the solute is in real suspension in the so-called solvent. In emulsoids, on the

other hand, we have two-phase liquids, each phase containing both components in different concentrations. The solute is able to combine to a certain extent with the solvent. They are intermediate between suspensions and true solutions. In certain cases there is evidence to show that the solute may be partially ionised. Solutions of native proteins, starch, dextrans, etc., are emulsoids, whereas the denaturised proteins behave more like suspensoids.

Sørensen's view as to the main differences between the two types of colloids is as follows :—

"The suspensoids show a viscosity differing but little from that of the pure external phase. There is generally a well-marked difference in electrical charge between the two phases. Only comparatively small concentrations of electrolytes are required to bring about coagulation, which is in most cases irreversible.

"The emulsoids show a great viscosity and power of foam formation; the system commonly does not show any marked difference in electrical charge between the two phases. Great concentrations of electrolytes are commonly necessary to bring about coagulation, which is in most cases reversible."

## B. Diffusion and Dialysis.

The difference between crystalloids and colloids that has been most emphasised is the disparity in the rate of diffusion of the two substances. If a solution of sodium chloride or glucose be separated from distilled water by means of a film of collodion, parchment paper, or gold beater's skin, the dissolved substance is found to pass through the membrane, the process being known as diffusion. If a colloidal solution be tested in the same way, it will be found to pass through either very slowly or not at all. In other words, the colloids are relatively indiffusible. This is sometimes employed as a convenient method of separating crystalloids from colloids, and is known as *dialysis*. It should be noted, however, that abrupt transitions are not common in nature, and that all emulsoids do diffuse through such membranes, though extremely slowly as compared to crystalloids.

1. **Preparation of collodion sacs for dialysis.** A convenient size is made by use of a large boiling tube ( $200 \times 15$  mm.). Into a clean, dry tube pour about 10 cc. of the collodion solution described

below. Pour this back into the stock, revolving the tube with the mouth downwards so that an even film is left adherent to the walls of the tube. Add another portion of collodion solution and repeat as before. Allow the film to dry, so that it does not stick to the finger. When this point is reached fill the tube with cold water. Cut round the rim of the tube with a knife, pour off the water, and carefully detach the membrane from the side of the tube. Allow water to run between the sac and the glass. By means of a glass rod with a spatulate end, and by traction and twisting, the sac can usually be removed from the glass tube. Fill the sac with water. It should be perfectly transparent. A cork, bored with a large hole, can be tied into the upper end, and by means of this it can be suspended in a jar of distilled water. The secret of success is to fill the tube with water at a particular moment, determined by trial on each specimen of collodion. If the water be added too soon the sac is opaque and feeble. If it be added too late, it is somewhat difficult to remove the film from the glass without damage. Sacs prepared in this way are very much better than those made of parchment paper. They should be kept wet, as on drying they become porous.

NOTE.—*Preparation of Collodion Solution.* To 3 gm. of gun cotton (pyroxylin) add 75 cc. of dry ether and allow to stand for 10 or 15 minutes in a flask closed with a cork. 25 cc. of absolute ethyl alcohol are then added, and the pyroxylin dissolves to a mobile fluid, which does not require filtration. It should be allowed to stand until all bubbles have disappeared.

2. **Dialysis.** Mix 2 per cent. starch paste (see Ex. 135) with about one-tenth of its volume of saturated ammonium sulphate solution. Place the mixture in a collodion sac and suspend this in water contained in a tall jar. Care must be taken to avoid spilling any of the mixture into the jar. Examine the "dialysate" (the fluid in the jar) for starch by the iodine test (Ex. 136) and for ammonium sulphate by means of barium chloride at the end of half an hour. The starch test will probably be negative, whilst the sulphate test will be positive. The dialysate should also be examined for starch after 2 to 7 days.

### C. Osmotic Pressure.

Certain membranes can be prepared which allow of the passage of water molecules, but do not allow dissolved substances to pass through them. Such membranes are

called "semi-permeable." If a dissolved substance, like glucose, be separated from water by means of a semi-permeable membrane, the sugar solution is diluted by water passing through the membrane. This process, the passage of water through a membrane into a solution, is known as "osmosis," and is to be carefully distinguished from "diffusion," the passage of a dissolved substance through a membrane. If the sugar solution be contained in a vessel connected to a manometer, and arrangements are made to keep the volume of the solution constant, it is found that the water passing into the vessel causes a rise of pressure. The final pressure reached is known as "the osmotic pressure" of the solution.

It is not necessary here to enter into the theories of osmotic pressure, but it is important to note that the osmotic pressure of a solution depends on the number of particles in a given volume, no matter whether these particles be ions, molecules, or aggregates of molecules. Thus the osmotic pressure of a dilute solution of sodium chloride is nearly double that of an equimolecular solution of glucose, because in dilute solution the sodium chloride is almost completely dissociated into its constituent ions. From these considerations it follows that the osmotic pressure of a colloidal solution is extremely low in comparison with that of a crystalloid of the same percentage, for the number of particles in a given volume of the colloidal solution is very small compared with that in the crystalloid solution.

For non-electrolytes it has been shewn by Van 't Hoff that Boyle's law for gases can be applied to solutions, if we substitute osmotic pressure for gas pressure. It has also been found that the Law of Charles is obeyed, namely, that at constant volume the pressure varies as the absolute temperature.

It follows that in dilute solution

$V.P. = R.T.$ ; where  $V$  = Volume;  $P$  = Osmotic Pressure;  
 $T$  = Temperature (absolute), and  $R$  = a Constant.

Moreover, it has been shewn by Van 't Hoff that  $R$  in the case of osmotic pressure has the same value as in the case of gases, that is, the solution exerts the same osmotic pressure as the pressure that the dissolved substance would exert if it were gasified at the same temperature and confined in the same volume as that of the solution. It follows that one gramme-molecule of a non-electrolyte will exert an osmotic pressure at  $0^{\circ}\text{C}$ . of 760 mm. of mercury when the volume of the solution is 22.4 litres.



If  $w$  grams of a substance be dissolved in  $V$  cc. of solvent at  $t^\circ$  C. and the osmotic pressure produced be  $P$  mm. of mercury, the volume at  $0^\circ$  C. and at 760 mm. mercury will be

$$\frac{V \times 273 \times P}{(273 + t) \times 760} = V_0.$$

Now  $V_0$  contains  $w$  grams of substance, so 22400 cc. contains

$$\frac{22400 \times w}{V_0} \text{ grms.}$$

Since this weight of substance produces a pressure of 760 mm. at  $0^\circ$  C. when in a volume of 22.4 litres it follows that it is the molecular weight of the substance.

Instead of the formula  $V.P. = R.T.$ , we must use the following for electrolytes,

$V.P. = \frac{2i + (100 - i)}{100} R.T.$ , where  $i$  is the percentage of the substance ionised.

3. **Chemical garden.** To a dilute aqueous solution of potassium ferrocyanide add a particle of solid ferric chloride. A film of Prussian blue is formed round the solid. This membrane is semipermeable, and allows water to pass in to dissolve the ferric chloride. The osmotic pressure of this solution being greater than that of the solution outside, water passes into the cell, which expands, and may assume remarkable forms.

4. A drop of a fairly strong solution of potassium ferrocyanide is added to a dilute solution of copper sulphate. A semipermeable cell of copper ferrocyanide is thus formed around the drop. The osmotic pressure of the potassium ferrocyanide being greater than that of the copper sulphate, pure water passes from the sulphate into the cell. This results in a concentration of copper sulphate immediately around the cell, and blue striae can be seen descending owing to the greater density of the strong copper sulphate solution thus formed.

#### D. Freezing Point.

The freezing point of a solution of a substance is always lower than that of the solvent. The depression of the freezing point ( $\Delta$ ) depends on the number of particles in a given volume of the solution. We have already seen that the osmotic pressure of a solution also varies with the number of particles in a given volume of the solution. It therefore follows that  $\Delta$  varies with the osmotic pressure.

Consequently the osmotic pressure of a solution is most conveniently estimated by a determination of its freezing point.

The depression of the freezing point ( $\Delta$ ) for a given concentration of a substance varies with the solvent employed, the relationship for non-electrolytes being  $\frac{\Delta}{w} \times M = C$ , where  $w$  is the weight of a substance of molecular weight  $M$  dissolved in 100 grams of the solvent and  $C$  is the "coefficient of depression" for the particular solvent. If  $s$  grms. of solvent are taken instead of 100, it follows that since  $\Delta$  is proportional to the concentration

$$\frac{s}{100} \times \frac{\Delta}{w} \times M = C.$$

The value of  $C$  for water is  $18.6^\circ \text{C.}$ : for acetic acid it is  $39^\circ \text{C.}$

Van 't Hoff has shewn that the value of  $C$  can be calculated from the formula  $C = \frac{2 T^2}{100 L}$ , where  $T$  is the absolute temperature of the freezing point, and  $L$  is the latent heat of fusion of the solvent.

Thus for water  $T = 273^\circ$

$$L = 80$$

$$\text{So } C = \frac{2 \times 273^2}{100 \times 80} = 18.6^\circ.$$

With non-electrolytes, therefore, the gramme-molecule in 1,000 gm. of water causes a depression ( $\Delta$ ) of  $1.86^\circ \text{C.}$

So that  $\frac{\Delta}{1.86} = \text{molecular concentration.}$

For electrolytes  $\frac{\Delta}{1.86} = \text{concentrations of (ions + molecules)}$ , so that if a substance be ionised to the extent of  $i$  per cent. the molecular concentration is

$$\frac{\Delta \times 100}{1.86 \times (2i + 100 - i)}.$$

The quantitative relationship between osmotic pressure and  $\Delta$  for aqueous solutions can be readily calculated as follows.

The gramme-molecule in 22.4 litres gives an osmotic pressure of 760 mm. of mercury at  $0^\circ \text{C}$ .

The gramme-molecule in 1 litre gives a  $\Delta$  of  $1.86^\circ \text{C}$ .

So the gramme-molecule in 22.4 litres gives a  $\Delta$  of  $\frac{1.86}{22.4} = 0.083^\circ \text{C}$ .

So a  $\Delta$  of  $0.083^\circ \text{C}$ . corresponds to an osmotic pressure of 760 mm.

So a  $\Delta$  of  $0.001^\circ \text{C}$ . corresponds to an osmotic pressure of 9.1 mm.

Thus a 5 per cent. solution of glucose (Mol.wt. = 180) has a  $\Delta$  of  $1.86 \times \frac{50}{180} = 0.517^\circ \text{C}$ ., and an osmotic pressure of  $51.7 \times 9.1 = 470$  mm. Hg.

The  $\Delta$  of Blood is about  $0.55^\circ \text{C}$ ., corresponding to an osmotic pressure of about 500 mm. Hg.

Owing to the relatively small number of particles in a given volume of a colloidal solution, it follows that the freezing point of such solutions is only very slightly lower than that of distilled water. Since it is very difficult to remove the last traces of electrolytes by dialysis, it is not easy to obtain reliable figures for the osmotic pressure of the colloids. Sørensen has recently investigated the problem and has been successful in overcoming the technical difficulties. He states that the osmotic pressure of crystalline egg-albumin indicates a molecular weight of 34,000.

**5. The determination of the freezing point by Beckmann's method. (Cryoscopy.)**

Take the freezing point of (a) distilled water; (b) M/5 NaCl (1.16 per cent.); (c) M/5 glucose (3.6 per cent.).

Use the apparatus shown in fig. 1. In the outer chamber (c) place a mixture of ice and water and solid sodium chloride, or a saturated solution of salt.

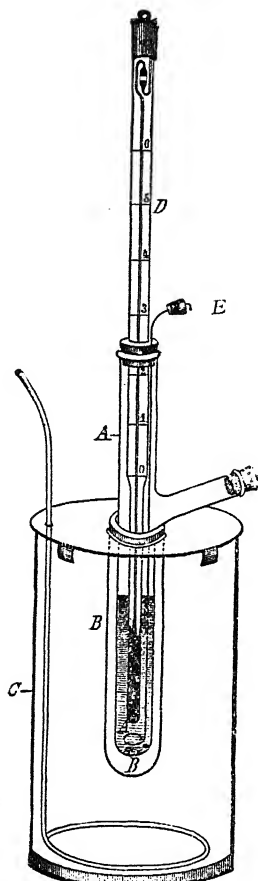


Fig. 1. Beckmann's freezing point apparatus.



Fig. 2. Beckmann's Thermometer.

In the tube A place enough distilled water to cover the bulb of the Beckmann thermometer D. This is graduated to  $1/100^{\circ}$  C. and can be read by means of a magnifying glass to  $1/1000^{\circ}$  C. The thermometer must not touch the sides or bottom of the tube A.

The tube B serves as an air jacket to A. Stir the water regularly by means of the (platinum) stirrer E. The temperature falls, and then after a time rises sharply, and remains steady for a considerable time. The temperature to be read is the highest obtained at this rise. This is the freezing point (W) of distilled water.

Now replace the water by the fluid, rinsing the tube out with it once or twice. Repeat the experiment and note the freezing point (F) as before.  $W - F = \Delta$ .

NOTES.—1. It is of the utmost importance to take care to prevent too great a super-cooling of the fluid. This should never exceed  $1^{\circ}\text{C}$ . If it has exceeded this in a preliminary experiment, it must be repeated, and when the temperature has fallen  $0.5^{\circ}\text{C}$ . below the freezing point, a minute crystal of ice must be introduced through the side tube. These crystals are best prepared by taking, in a dry test-tube, some hollow glass beads (that have been carefully dried), adding a small amount of the fluid, pouring off the excess, and immersing the tube in a freezing mixture. They should be introduced by means of a pair of cooled forceps.

2 The observed  $\Delta$  is usually too great, owing to the super-cooling. The simplest correction is

$$\Delta \text{ corrected} = \Delta \text{ observed} \times \left(1 - \frac{C}{80}\right)$$

where C = the super-cooling in degrees.

3. To set the thermometer. Turn the thermometer upside down, and by gentle shaking mix the mercury in the upper portion with that in the capillary tube. Then place the thermometer in water at about  $2^{\circ}\text{C}$ . Give a slight knock, and thus break the mercury column. It is now ready for use.

4. When reading the thermometer during an experiment it should be tapped with a piece of indiarubber tubing.

### E. The electrical properties of colloids.

Under certain conditions it is found that colloidal particles carry an electric charge. In some cases they exhibit electrical conductivity, due to the fact that the substances are partially ionised. But even if they do not exhibit this phenomenon it is often found that they tend to move towards one of the poles when a strong (100 volts) constant current is sent through the solution. In some cases this movement ("kataphoresis") is towards the anode, *i.e.* the particles carry a negative charge; in other cases it is towards the kathode. It is important to note that the direction of the migration can be changed in many cases by varying the reaction of the fluid in which the colloid is suspended. Thus metaproteins, albumins, etc., carry a

positive charge in acid solution and a negative charge in alkaline solution. At some particular reaction they seem to be electrically neutral, *i.e.* kataphoresis cannot be observed. This reaction is known as "the iso-electric point" of the particular colloid. It is discussed in more detail on p. 31.

#### F. The precipitation of colloids.

Colloids, as we have seen, are two-phase solutions. One, the solid, phase contains a high concentration of the solute and a low concentration of the solvent: the other, the liquid, phase contains a low concentration of the solute in the solvent. By certain changes in the conditions the solid phase can be dehydrated, so that the solution may become opalescent. An increase of this effect may result in the formation of particles visible to the naked eye, or even a dense precipitate that can, in some cases, be removed completely by filtration. In some cases this precipitate can be "dissolved" or "dispersed" by reverting to the original conditions. In other cases the change is irreversible, the material having been "coagulated."

It is impossible to discuss fully the various conditions that tend to cause aggregation (*i.e.* precipitation) on the one hand, or dispersion (*i.e.* solution) on the other, since they vary considerably with different colloids. But it is important to note that many cases can be explained fairly satisfactorily on the theory that the dispersed or dissolved condition of a colloid is due to the fact that it carries an electric charge, the removal of which causes precipitation.

Some examples of this are given below:—

##### (a) *By colloids with an opposite electrical charge.*

If ferric chloride be thoroughly dialysed a colloidal suspension of ferric hydroxide is obtained, generally known as "dialysed iron." This carries a positive charge. If this be added to certain albumins which carry a negative charge the two colloids mutually precipitate one another. This gives us a valuable method for removing certain proteins from solution. (See Ex. 310, p. 350.)

(b) *By changing the reaction of the fluid.*

In acid solution most colloids "adsorb" the positively charged and readily diffusible hydrogen ions and acquire a positive charge. In alkaline solutions they adsorb hydroxyl ions and become negative. Many proteins are therefore soluble both in acids and alkalies, but at some particular reaction of the fluid they adsorb equal numbers of H and OH ions, lose their charge, and are precipitated. The exact reaction at which this takes place varies with different colloids, and is the above-mentioned iso-electric point. Another way of explaining this phenomenon will be found on p. 31.

6. **The determination of the iso-electric point of casein.**

Into a 50 cc. measuring flask place 0.3 gm. of pure casein (Hammersten's). Add about 25 cc. of distilled water, previously warmed to about 40 C. and exactly 5 cc. of N. sodium hydroxide. Agitate till the casein dissolves, taking care to prevent frothing. Rapidly add 5 cc. of N. acetic acid, mix, cool, and make up to 50 cc. with distilled water. A faintly opalescent solution of casein in 0.1 N. sodium acetate is thus obtained.

Make up the following series of tubes, using clean dry test-tubes.

| Tube No.                             | 1    | 2    | 3    | 4   | 5 | 6 | 6 | 8 | 9   |
|--------------------------------------|------|------|------|-----|---|---|---|---|-----|
| cc. Casein in 0.1 N. sod. acetate .. | 1    | 1    | 1    | 1   | 1 | 1 | 1 | 1 | 1   |
| cc. Distilled water ..               | 8.38 | 7.75 | 8.75 | 8.5 | 8 | 7 | 5 | 1 | 7.4 |
| cc. 0.01 N. acetic acid              | 0.62 | 1.25 |      |     |   |   |   |   |     |
| cc. 0.1 N. acetic acid ..            |      |      | 0.25 | 0.5 | 1 | 2 | 4 | 8 |     |
| N. acetic acid ..                    |      |      |      |     |   |   |   |   | 1.6 |

Place the casein solution in the tubes first, then the water, and mix. Now add the acetic acid to the first tube and shake immediately. Then add the acid to the second tube and shake this, and so on. Examine the tubes at intervals and record observations as below.

o = no change.      + = opalescence.      × = precipitate.

| Tube No.          | 1 | 2 | 3 | 4   | 5   | 6  | 7  | 8 | 9 |
|-------------------|---|---|---|-----|-----|----|----|---|---|
| On mixing ..      | 0 | 0 | + | ++  | +++ | ++ | +  | + | 0 |
| After 10 mins. .. | 0 | 0 | + | +++ | xxx | xx | ++ | + | 0 |
| After 20 mins. .. | 0 | 0 | + | x   | xxx | xx | ++ | + | 0 |

The precipitation is greatest in tube 5.

The concentration of hydrogen ions (see p. 19) can be calculated approximately from the following formula, no allowance being made for the acidity of the casein.

$$(H) = \frac{K (\text{acetic acid in mols. per litre})}{\alpha (\text{sodium acetate mols. per litre})}$$

(H) = Hydrogen ions in grams per litre.

K = dissociation constant of acetic acid =  $1.85 \times 10^{-5}$ .

$\alpha$  = degree of dissociation of sodium acetate. 0.87 for 0.01 N.  
0.79 for 0.1 N.

The theoretical basis for the formula is given on p. 19.

Thus in tube 5,

$$\frac{(H) = 1.85 \times 10^{-5} \times 10^{-2}}{0.87 \times 10^{-2}} = 2.13 \times 10^{-5}.$$

Below are the (H) and  $P_H$  (see p. 16) of the various tubes.

| Tube | (H)                   | $P_H$ | Tube | (H)                   | $P_H$ |
|------|-----------------------|-------|------|-----------------------|-------|
| 1    | $1.32 \times 10^{-6}$ | 5.88  | 6    | $4.26 \times 10^{-5}$ | 4.37  |
| 2    | $2.66 \times 10^{-6}$ | 5.75  | 7    | $8.52 \times 10^{-5}$ | 4.07  |
| 3    | $5.32 \times 10^{-6}$ | 5.27  | 8    | $1.70 \times 10^{-4}$ | 3.77  |
| 4    | $1.06 \times 10^{-5}$ | 4.97  | 9    | $3.40 \times 10^{-4}$ | 3.47  |
| 5    | $2.13 \times 10^{-5}$ | 4.67  |      |                       |       |

Still finer adjustments of the reaction can be obtained by suitably varying the concentration of acetic acid. The (H) can be calculated from the formula.



*(c) By the addition of neutral salts.*

If a suspensoid carries a negative charge it exerts an attraction for positively charged ions (kations). The adsorption of these by the colloid may cause a neutralisation of the charge, and therefore precipitation. In such cases it is found that a bi- or tri-valent ion is very much more potent than a monovalent ion. Thus, if a colloid is negatively charged it may be readily precipitated by  $\text{BaCl}_2$ ; if it carries a positive charge it may be readily precipitated by  $\text{Na}_2\text{SO}_4$ . The precipitation of an emulsoid by a large excess of neutral salt, such as by saturation with ammonium sulphate, is probably a different phenomenon.

**7. The precipitating effect of various ions on colloids.**

Prepare a solution of casein in 0.1 N. sodium acetate as described in Ex. 6.

To 2 cc. add 17.5 cc. of distilled water, and then 0.5 cc. of 0.1 N. acetic acid and mix quickly. A solution of casein is thus obtained, alkaline to the iso-electric point, and therefore carrying a negative charge. Divide the solution into four equal parts and place them into four clean tubes labelled -1, -2, -3 and -4. To another 2 cc. of the original solution of casein add 10 cc. of distilled water and 8 cc. of 0.1 N. acetic acid, and mix quickly. An acid solution of casein is thus obtained. Divide into four parts and place them into four clean tubes labelled +1, +2, +3 and +4.

To the tubes marked 1 add 3 drops of N. KCl (7.45 per cent.).

To the tubes marked 2 add 1 drop of N.  $\text{BaCl}_2$  (10.4 per cent.).

To the tubes marked 3 add 1 drop of N.  $\text{K}_2\text{SO}_4$  (8.7 per cent.).

Mix the contents of each tube and place the set of 8 tubes in a water bath at about 35 C. Examine them after 15 minutes, recording the results as in the previous exercise.

|   | -      | +  |
|---|--------|----|
| 1 | +      | ++ |
| 2 | x      | +  |
| 3 | o or + | xx |
| 4 | o      | o  |

It will be noted that the electro-negative colloid is most readily precipitated by  $\text{BaCl}_2$ , which contains a di-valent positive ion. The electro-positive colloid is most readily precipitated by  $\text{K}_2\text{SO}_4$ , which contains a di-valent negative ion.

The tubes may now be warmed to  $60^\circ \text{C.}$ , and the further effect noted.

(d) *By the addition of compounds with complex ions.*

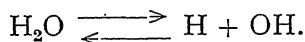
It is found that colloids that carry a positive charge are often readily precipitated by compounds with a complex negative ion. Thus, proteins in acid solution generally carry a positive charge, and they are precipitated by phosphotungstic, phosphomolybdic, tannic, or ferrocyanic acids. Probably the complex ions are more readily adsorbed by the positively charged colloid than are the simple ions. The charge of the colloid thus being neutralised, precipitation of the complex takes place.

If the colloid carries a negative charge it is often readily precipitated by compounds with a complex positive ion, such as the hydrochlorides of the alkaloids, aromatic bases, etc.

### G. The Concentration of Hydrogen ions.

The only satisfactory method of expressing the "reaction" of a fluid is in terms of the concentration of hydrogen ions per litre of the fluid. This concentration is so important as a factor in the physiological properties of fluids that the theory of the matter should be grasped by students at an early stage of their physiological studies.

Pure distilled water is very slightly ionised into hydrogen ions or hydriions and hydroxyl ions or hydroxidions.



This dissociation proceeds to an equilibrium, in which, according to the laws of mass action,

$$\frac{(\text{H}) \times (\text{OH})}{(\text{H}_2\text{O})} = \text{a constant.}$$

So  $(H) \times (OH) = \text{a constant} \times (H_2O).$

The brackets indicate the concentration per litre of gram-ions or gram-moles respectively.

Since the mass of undissociated water is enormously large compared to the mass of the free ions, it can be regarded as a constant, so

$$(H) \times (OH) = \text{a constant.}$$

This constant varies considerably with the temperature. At  $21^\circ \text{C.}$  it is  $10^{-14}$ . Since the ions are equal in number, each has a concentration of  $10^{-7}$  gram-ions per litre. It must be particularly noticed that the product and not the sum of the gram-ion concentrations is constant.

If an acid be added to distilled water the acid is partially or completely dissociated into hydrogen ions, and the negative ions characteristic of the acid employed. In such a mixture the concentration of hydrogen ions per litre at  $21^\circ \text{C.}$  is greater than  $10^{-7}$  grams, and the solution is "acid." If  $(H)$  be increased to  $10^{-4}$  it follows that the concentration of hydroxyl ions per litre must be decreased to  $10^{-10}$  gram-ions. For  $(H) \times (OH) = 10^{-14}$ . If an alkali be added to distilled water the base is dissociated into hydroxyl ions and certain positive ions. The concentration of hydrogen ions per litre at  $21^\circ \text{C.}$  is consequently less than  $10^{-7}$  grams, and the solution is "alkaline."

A "neutral" solution is one in which  $(H)$  at  $21^\circ \text{C.} = 10^{-7}$ .

An "acid" solution is one in which  $(H)$  at  $21^\circ \text{C.}$  is greater than  $10^{-7}$ .

An "alkaline" solution is one in which  $(H)$  at  $21^\circ \text{C.}$  is less than  $10^{-7}$ .

Acids differ markedly in the degree to which they are ionised in solution. "Strong" acids, like  $\text{HCl}$  or  $\text{HNO}_3$ ,

are freely ionised ; whilst " weak " acids, like acetic acid, are only feebly ionised.

By electrical measurements of the conductivity of the solution it has been shewn that 0.1 N.HCl is ionised to the extent of 84 per cent. at 18° C. If it were completely ionised there would be 0.1 gm. of hydrion per litre. As it is only partially ionised,

$$(H) \text{ is } 0.1 \times \frac{84}{100} = 0.084 = 8.4 \times 10^{-2} \text{ at } 18^{\circ} \text{ C.}$$

Similarly, 0.1 N. acetic acid is only dissociated to the extent of 1.36 per cent. So in this case

$$(H) = 0.1 \times \frac{1.36}{100} = 0.00136 = 1.36 \times 10^{-3}.$$

This method of expressing the hydrogen ion concentration is not convenient. It is preferable to adopt the notation of Sørensen, who introduced the symbol  $P_H$  to denote the "hydrogen-ion-exponent."  $P_H$  is the logarithm to the base 10 of (H), the negative sign being omitted. In other words

$$P_H = -\log_{10} (H).$$

A few examples should make its meaning clear.

0.1 N.HCl has  $(H) = 8.4 \times 10^{-2}$ . Now  $\log_{10} 8.4 = 0.92$ .

So  $8.4 \times 10^{-2} = 10^{0.92-2} = 10^{-1.08}$ . So  $P_H = 1.08$ .

0.1 N. acetic acid has

$$(H) = 1.36 \times 10^{-3} = 10^{0.133-3} = 10^{-2.867}.$$

So

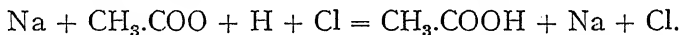
$$P_H = 2.867.$$

It will be observed that  $P_H$  decreases as the acidity increases. Also that if (H) is doubled,  $P_H$  is not halved, but only decreased by 0.301, since  $\log_{10} 2 = 0.301$ .

It is important to note that the  $P_H$  of a solution cannot be determined by the ordinary method of titration. Let us consider the case of 0.1 N.HCl and 0.1 N. acetic acid. If these be titrated with 0.1 N.NaOH until they each give a pink with phenol phthalein, 10 cc. of each acid will require exactly 10 cc. of the alkali, and will therefore

have apparently the same acidity. But actually the hydrochloric acid has an (H) over 60 times greater than the acetic acid. The reason for this is that the acetic acid is only very slightly ionised, the amount ionised being a certain proportion of the total acid present. As soon as the ionised part has been removed by the addition of a base, a further fraction of the previously undissociated acid is ionised. This process is repeated with further additions of alkali until the whole of the acid originally present has become dissociated, and its hydrogen ions have united with the hydroxyl ions of the base. The next trace of added alkali reacts with the indicator to give a pink colour. Titration therefore only gives us an index of the *capacity* of the solution to neutralise acids or alkalis ; it does not give us information concerning the *potential* of the hydrogen ions, *i.e.* the  $P_H$ .

"**Buffers.**" A single drop of 0.02 N.HCl added to a litre of pure water at 18° C. would cause a change in  $P_H$  from 7.07 to about 6. A trace of diffusible alkali from a glass bottle might change the  $P_H$  to 8, or even higher, whereas exposure to the  $CO_2$  of the air might cause a drop to about 6. Thus it is extremely difficult to maintain any constancy of  $P_H$  in such a solution. But with certain substances present the addition of a small amount of acid or alkali causes only a minimal change in  $P_H$ . Such substances are called "Buffers." Various solutions are used for this purpose, such as phosphates, citrates, borates, and acetates. Let us consider the case of a solution of sodium acetate, to which is added a small amount of hydrochloric acid. Both substances are freely dissociated so that the following ions are originally present, Na,  $CH_3.COO$ , H, Cl. Now acetic acid is a weak acid, which means that  $CH_3.COO$  and H ions can exist together only in very low concentrations. We therefore get



Thus the H ions of the added hydrochloric acid nearly disappear, owing to the presence of the buffer sodium

acetate. It must be noted that they do not all disappear, for some of the acetic acid formed is dissociated into H and  $\text{CH}_3\text{COO}$ .

In the animal body the proteins, sodium bicarbonate, and phosphates all function as buffers, and help to maintain a constancy in the hydrogen ion concentration of the tissue fluids.

**The effect of dilution on (H).** With a weak acid of the type HA, the extent of dissociation is governed by the equation

$$\frac{(\text{H}) \times (\text{A})}{(\text{HA})} = K \quad \text{---} \quad (1)$$

(HA) is the concentration of the undissociated molecules per litre, and K is the "Dissociation constant" of the acid.

Since  $(\text{H}) = (\text{A})$ , we can write this

$$(\text{H})^2 = K(\text{HA}) \quad \text{or} \quad (\text{H}) = \sqrt{K(\text{HA})}.$$

This indicates that if a solution of a weak acid be diluted four times (H) is halved; if it be diluted 16 times it is reduced to one-fourth.

In the presence of any considerable amount of the sodium salt, the effect of dilution is quite different.

We can write equation (1) in the following form:

$$(\text{H}) = \frac{K(\text{HA})}{(\text{A})} \quad \text{---} \quad (2)$$

The sodium salts of weak acids are very freely dissociated, so that there is a relatively high concentration of A ions in the solution of the mixture. From equation (2) it will be seen that an increase of (A) must cause a decrease in (H). The dissociation of the weak acid being thus depressed it follows that practically all the acid is present in the undissociated form, so that we can assume that  $(\text{HA}) = (\text{acid})$ . Further, practically all the free ions arise from the dissociation of the sodium salt, so that  $(\text{A}) = (\text{Sodium salt})$ .

We can therefore write equation (2) as

$$(H) = \frac{K(\text{acid})}{(\text{Sodium salt})} \quad \text{---} \quad (3)$$

Since the sodium salt is not fully dissociated, except in high dilutions, it is more correct to write it

$$(H) = \frac{K(\text{acid})}{\alpha (\text{Sodium salt})} \quad \text{---} \quad (4)$$

where  $\alpha$  is the degree of dissociation of the salt.

It follows from this that the  $(H)$  of such a mixture is mainly conditioned by the relative concentrations of the acid and of its salt, and is only very slightly affected by dilution, which does not alter the *relative* concentrations. This is of considerable importance, since a large number of physiological fluids can be regarded as mixtures of weak acids with their sodium or potassium salts, and so suffer little change in  $(H)$  on dilution.

### The determination of the hydrogen ion concentration.

The most accurate method is an electrical one, involving expensive and intricate apparatus. It is too complicated to be described here. A valuable method that does not require elaborate apparatus is the "indicator," or "colorimetric" method.

An *indicator* is a substance that varies in colour tone or in depth of colour with the  $P_H$  of the solution. Each indicator shows a colour change over a certain range of  $P_H$ . At some particular  $P_H$  the indicator may show an intermediate or faint tint. The solution is then said to be "neutral" to this indicator. It does not follow that the solution is neutral in the strict sense, *i.e.*  $(H) = (OH)$ . The  $P_H$  of a solution "neutral to phenol phthalein" is about 9; that of a solution "neutral to methyl orange" is about 4, the  $(H)$  in the latter case being 100,000 times greater than in the former.

The method adopted for the determination of  $P_H$  by indicators is to take standard solutions of certain substances

which can be mixed in various proportions to give a series of solutions of a known  $P_H$ , which have been accurately determined by the electrical method. A given amount of a suitable indicator is added to a measured volume of the fluid, and also to equal volumes of the standard test solutions, contained in tubes or vessels as uniform as possible. The solutions that give exactly the same tints have the same hydrogen ion concentration, provided that this concentration is in the range of the indicator employed. The results are not as accurate as the electrical method, owing to the difficulty of exactly matching the tints and

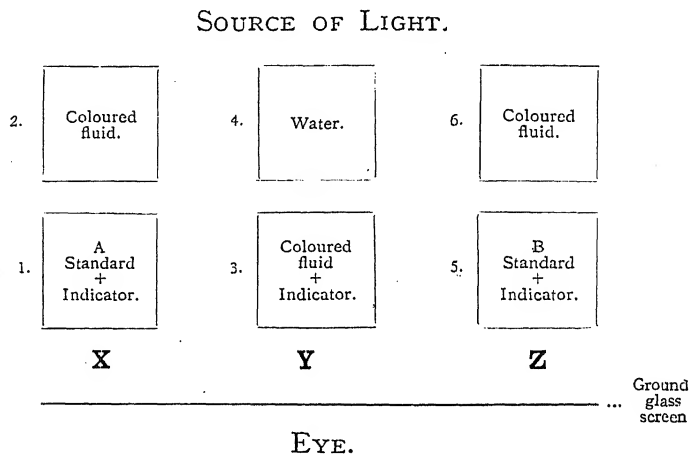


Fig. 3. Plan of arrangement of tubes in Cole and Onslow's Comparator.

also of the effect of proteins, salts, and other substances on the colour developed. If the fluid be coloured it is obvious that this simple method can only give very approximate results. Walpole overcame the difficulty by viewing the (*standard solution + indicator*) through a layer of the coloured fluid of the same depth as that of the (*coloured fluid + indicator*). A special instrument was devised for this purpose. Hurwitz, Meyer and Ostenberg used Walpole's principle, but employed test tubes held in a box



or "comparator." Cole and Onslow somewhat improved this by using a comparator containing three pairs of tubes, the arrangement being diagrammatically shown in fig. 3. The addition of a ground glass plate fixed to the comparator between the eye and the tubes has been found by the author very much to improve the apparatus, slight differences in colour being more readily detected.

The standard solutions taken are such that the appearance seen through Y is either intermediate between that seen through X and Z, or identical with one of them. The colour changes, and the ranges of the most useful indicators are given below in Table I. Certain other interesting data are presented in chart form in Table II.

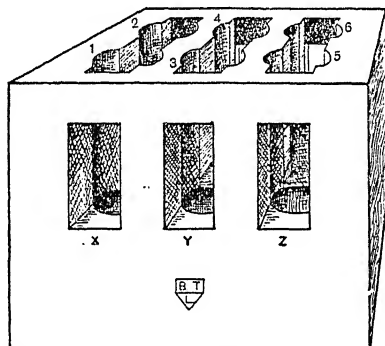


Fig 4. Cole and Onslow's Comparator.

The author can very strongly recommend the new sulphone-phthalein indicators introduced by Mansfield Clark, Lubs and Acree. For further information on the subject of the colorimetric method of determination of  $P_H$  the student is referred to an important series of papers by Clark and Lubs, "Journal of Bacteriology," Baltimore, Vol. II., pp. 1, 109 and 191 (1917).

TABLE I.

**Indicators.**

Those printed in **heavy type** are the most useful for ordinary work.

| TRADE NAME                     | CHEMICAL COMPOSITION                                   | RANGE OF $P_H$ | COLOUR CHANGE, Acid—alkaline. |
|--------------------------------|--|----------------|-------------------------------|
| 1. Methyl violet               |  | 0.1 to 3.2     | Green-blue                    |
| 2. <b>Thymol blue</b>          | Thymol - sulphone - phthalein                          | 1.2 to 2.8     | Red-yellow                    |
| 3. Toepfer's reagent           | Di - methyl - amino - azo-benzene                      | 2.9 to 4.2     | Red-yellow                    |
| 4. <b>Brom-phenol-blue</b>     | Tetra - brom - phenol - sulphone-phthalein             | 2.8 to 4.6     | Yellow-blue                   |
| 5. Methyl orange               | p - benzene - sulphonic acid-azo-di-methyl-aniline     | 3.1 to 4.4     | Red-yellow                    |
| 6. Congo red                   |  | 3.0 to 4.5     | Blue-red                      |
| 7. <b>Methyl-red</b>           | p - dimethyl amino - azo - benzene - o - carbonic acid | 4.4 to 6.0     | Red-yellow                    |
| 8. <b>Brom - cresol-purple</b> | Di - brom - o - cresol - sulphone-phthalein            | 5.2 to 6.8     | Yellow-purple                 |
| 9. Litmus                      |  | 5.4 to 7.8     | Red-blue                      |
| 10. <b>Brom-thymol blue</b>    | Di - brom - thymol - sulphone - phthalein              | 6.0 to 7.6     | Yellow-blue                   |
| 11. Neutral red                |  | 6.8 to 8.0     | Red-yellow                    |
| 12. <b>Phenol-red</b>          | Phenol-sulphone-phthalein                              | 6.8 to 8.4     | Yellow-red                    |
| 13. <b>Cresol-red</b>          | o - cresol - sulphone - phthalein                      | 7.2 to 8.8     | Yellow-red                    |
| 14. <b>Thymol-blue</b>         | Thymol - sulphone - phthalein                          | 8.0 to 9.6     | Yellow-blue                   |
| 15. Phenol phthalein           | Phenol phthalein                                       | 8.3 to 10.0    | Colourless-red                |
| 16. Thymol-phthalein           | Thymol-phthalein                                       | 9.3 to 10.5    | Colourless-blue               |

*Preparation of solutions.*

These are best prepared from standardised solutions of known strength.\* The most convenient concentrations for ordinary work are

0.04 per cent. for (1), (2), (4), (8), (10), and (14).

0.02 per cent. for (6), (12), and (13).

0.01 per cent. for (5).

0.02 per cent. in 60 per cent. alcohol for (3), (7) and (11).

0.04 per cent. in 60 per cent. alcohol for (15) and (16).

Strong aqueous solution, dialysed against distilled water for (9).

*Volume required.* In most cases ten drops to 10 cc. of the solution are about right. But the amount varies with the range, colour of solution, etc. Thus 12 drops of no. (12) may be required at  $P_H = 6.9$ , and only 5 drops at  $P_H = 8.0$ . It is essential that exactly the same amount be added to the measured volume of the fluid and to the same measured volumes of the standard solutions. The most convenient and accurate method of adding the drops is to have the bottle of indicators fitted with rubber corks pierced with Dreyer's dropping pipettes (fig. 5).

*Colour filters for dichroic indicators.* Bromophenol blue and brom-cresol purple are dichroic. To get reliable results, especially with turbid fluids, it is necessary to compare the solutions by using a source of light from which the blue rays have been screened off. This can be done by painting a piece of transparent tracing paper with a strong acid solution of phenol red, prepared by mixing one part of the stock 0.6 per cent. solution with one part of the standard 0.2 M acid potassium phosphate. The paper, while still wet, is pinned across the front of a box containing one or two powerful carbon filament lamps. The ex-

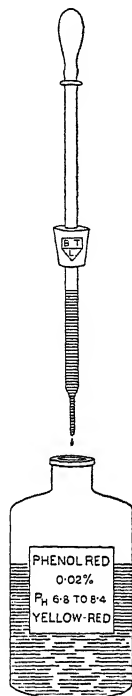


Fig. 5. Bottle and Dreyer's Dropping Pipette for Indicator Solutions.

\* These can be obtained from Messrs Baird & Tatlock, 14, Cross Street, Hatton Gardens, E.C.

amination should be conducted in a dark room, or the external light should be cut off by using a dark cloth.

Another method is to take an unexposed photographic plate, fix it in hypo in a dark room, wash for some hours in running water, stain by immersion in the dye, drying and mounting a piece on the comparator on the side towards the light, in place of the ground glass screen.

#### Standard solutions of definite $P_H$ .

The most convenient sets of solutions that have been worked out are those of Clark and Lubs. A constant volume (50 cc.) of a standard solution of acid potassium phthalate, acid potassium phosphate or of boric acid is measured into a 200 cc. measuring flask. A given amount ( $x$  cc.) of standard NaOH or HCl is then added, and the volume brought to the mark with distilled water. The  $P_H$  obtained with the different solutions are given in the tables below. If intermediate points are desired, they can be obtained from curves drawn from the points given.

#### *Preparation of solutions.*

*0.2 M acid potassium phthalate.* Dissolve 40.828 gm. in distilled water and make up to 1 litre. The salt should be recrystallised from distilled water and dried at  $110^\circ$  C. for some hours.

*0.2 M acid potassium phosphate.* Dissolve 27.231 gm. in distilled water and make up to 1 litre. The salt should be recrystallised from distilled water and dried at  $110^\circ$  C. for some hours.

*0.2 M Boric Acid in 0.2 KCl.* Dissolve 12.4048 gm. of air dried boric acid and 14.912 gm. pure ignited KCl in distilled water and make up to 1 litre.

#### *Sodium Hydroxide.*

Dissolve 100 grams of the best NaOH in 100 cc. of distilled water in an Erlenmeyer flask of resistance glass. Cover the mouth of the flask with tin foil, and allow the solution to stand overnight till the carbonate has mostly settled. Cut a hardened filter paper to fit a Buchner funnel. It must be noted that the paper shrinks a little in treatment, and must be cut rather large. Treat it with warm, strong [1:1] NaOH solution. Decant the soda and wash the paper first with absolute alcohol, then with dilute alcohol and finally with large quantities of distilled water. Place

the paper on the Buchner funnel and apply gentle suction until the greater part of the water has evaporated. Now pour the concentrated alkali upon the middle of the paper, spread it with a glass rod, and filter under suction. The clear solution is now diluted quickly with cold distilled water, that has been recently boiled to remove  $\text{CO}_2$ , to make approximately  $N$ .  $\text{NaOH}$  (10 cc. per litre makes about  $0.2 N$ ). 10 cc. of this is withdrawn and roughly standardised against  $N$ .  $\text{HCl}$ . It is then diluted till it is approximately  $0.2 N$  with  $\text{CO}_2$ -free water and the solution poured into a paraffined bottle, to which a burette and soda-lime guard tubes have been attached (see fig. 6). The solution is then accurately standardised against weighed amounts of the pure acid potassium phthalate. To do this accurately weigh up about 1.5 gms. of the salt, dissolve in about 30 cc. of distilled water, add phenol phthalein and titrate with the alkali till a faint but distinct and permanent pink is developed. A current of  $\text{CO}_2$ -free air should be blown through the solution during the titration.

The apparatus shewn in fig. 35 is convenient for this purpose.

If  $p$  be the exact weight of the phthalate taken, and  $s$  the volume of soda required, the normality of the soda is

$$\frac{1,000 \times p}{204.14 \times s} = a.$$

Instead of using  $x$  cc. of  $0.2 N$ , the amount of the standardised soda that must be employed is

$$\frac{x \times 0.2}{a} \text{ cc.}$$

It is convenient to label the bottle with the factor  $\frac{0.2}{a}$ .

*0.2 N Hydrochloric acid.* This can be prepared from a freshly distilled 20 per cent. solution, and standardised against the standard soda, using methyl red as the indicator.

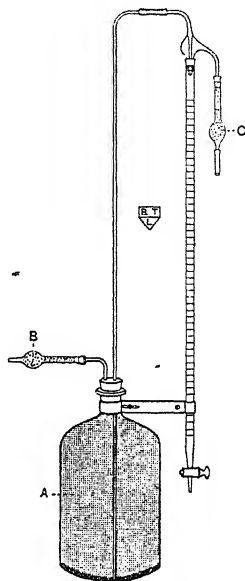


Fig. 6. Paraffined bottle (A) for storing standard alkali. B and C are soda lime tubes. The burette is filled by sucking at C.

*Series A.* 50 cc. 0.2 M. acid potassium phthalate.  
 $x$  cc. 0.2 N. HCl.  
 Diluted to 200 cc.

Indicators recommended { Thymol blue.  
 Brom-phenol blue.

| $P_H$ | $x$   | $P_H$ | $x$   | $P_H$ | $x$  |
|-------|-------|-------|-------|-------|------|
| 2.2   | 46.70 | 2.9   | 22.80 | 3.6   | 5.97 |
| 2.3   | 42.50 | 3.0   | 20.32 | 3.7   | 4.30 |
| 2.4   | 39.60 | 3.1   | 17.70 | 3.8   | 2.63 |
| 2.5   | 37.00 | 3.2   | 14.70 | 3.9   | 1.00 |
| 2.6   | 32.95 | 3.3   | 11.80 |       |      |
| 2.7   | 29.60 | 3.4   | 9.90  |       |      |
| 2.8   | 26.42 | 3.5   | 7.50  |       |      |

*Series B.* 50 cc. 0.2 M. acid potassium phthalate.  
 $x$  cc 0.2 N. NaOH.  
 Diluted to 200 cc.

Indicators recommended { Brom-phenol blue.  
 Methyl red.  
 Brom-cresol purple.

| $P_H$ | $x$   | $P_H$ | $x$   | $P_H$ | $x$   |
|-------|-------|-------|-------|-------|-------|
| 4.0   | 0.40  | 4.8   | 17.70 | 5.6   | 39.85 |
| 4.1   | 2.20  | 4.9   | 20.95 | 5.7   | 41.90 |
| 4.2   | 3.70  | 5.0   | 23.85 | 5.8   | 43.00 |
| 4.3   | 5.17  | 5.1   | 27.20 | 5.9   | 44.55 |
| 4.4   | 7.50  | 5.2   | 29.95 | 6.0   | 45.45 |
| 4.5   | 9.60  | 5.3   | 32.50 | 6.1   | 46.20 |
| 4.6   | 12.15 | 5.4   | 35.45 | 6.2   | 47.00 |
| 4.7   | 14.60 | 5.5   | 37.70 | 6.3   | 48.10 |

*Series C.* 50 cc. 0.2 M. acid potassium phosphate.

$x$  cc. 0.2 N. sodium hydroxide.

Diluted to 200 cc.

Indicators recommended { Brom-cresol purple.  
Brom-thymol blue.  
Phenol red.

| P <sub>H</sub> | $x$   | P <sub>H</sub> | $x$   | P <sub>H</sub> | $x$   |
|----------------|-------|----------------|-------|----------------|-------|
| 5.8            | 3.72  | 6.6            | 17.80 | 7.4            | 39.50 |
| 5.9            | 4.70  | 6.7            | 21.00 | 7.5            | 41.20 |
| 6.0            | 5.70  | 6.8            | 23.65 | 7.6            | 42.80 |
| 6.1            | 7.40  | 6.9            | 26.50 | 7.7            | 44.20 |
| 6.2            | 8.60  | 7.0            | 29.63 | 7.8            | 45.20 |
| 6.3            | 10.19 | 7.1            | 32.50 | 7.9            | 46.00 |
| 6.4            | 12.60 | 7.2            | 35.00 | 8.0            | 46.80 |
| 6.5            | 16.00 | 7.3            | 37.40 |                |       |

*Series D.* 50 cc. 0.2 M. boric acid in 0.2 M. potassium chloride.

$x$  cc. 0.2 N. sodium hydroxide.

Diluted to 200 cc.

Indicators recommended { Cresol red.  
Thymol blue.

| P <sub>H</sub> | $x$  | P <sub>H</sub> | $x$   | P <sub>H</sub> | $x$   | P <sub>H</sub> | $x$   |
|----------------|------|----------------|-------|----------------|-------|----------------|-------|
| 7.8            | 2.61 | 8.4            | 8.50  | 9.0            | 21.30 | 9.6            | 36.85 |
| 7.9            | 3.30 | 8.5            | 10.40 | 9.1            | 24.30 | 9.7            | 39.00 |
| 8.0            | 3.97 | 8.6            | 12.00 | 9.2            | 26.70 | 9.8            | 40.80 |
| 8.1            | 4.80 | 8.7            | 14.30 | 9.3            | 29.95 | 9.9            | 42.50 |
| 8.2            | 5.90 | 8.8            | 16.30 | 9.4            | 32.00 | 10.0           | 43.90 |
| 8.3            | 7.30 | 8.9            | 19.00 | 9.5            | 34.50 |                |       |

*Series E. Sodium acetate and acetic acid.*

The following series is given as being convenient for certain experiments. It should be noted that the  $P_H$  of the solutions is only very slightly changed by considerable dilution with water.\*

*Preparation of Solutions.*

N. acetic acid is prepared by titration against N. soda. 0.2 N. acetic acid is prepared from this by diluting 200 cc. to 1000 cc. with distilled water. 0.2 N. sodium acetate is prepared by mixing 200 cc. of the N. acetic acid with 200 cc. of the N. soda employed and diluting to 1000 cc. with distilled water.

Take  $x$  cc. of the sodium acetate, and add  $(10-x)$  cc. of the 0.2 N. acetic acid.

| $P_H$ | $x$  | $(10-x)$ | $P_H$ | $x$  | $(10-x)$ |
|-------|------|----------|-------|------|----------|
| 3.8   | 1.2  | 8.8      | 4.8   | 5.95 | 4.05     |
| 3.9   | 1.5  | 8.5      | 4.9   | 6.5  | 3.5      |
| 4.0   | 1.8  | 8.2      | 5.0   | 7.0  | 3.0      |
| 4.1   | 2.2  | 7.8      | 5.1   | 7.45 | 2.55     |
| 4.2   | 2.65 | 7.35     | 5.2   | 7.85 | 2.15     |
| 4.3   | 3.1  | 6.9      | 5.3   | 8.25 | 1.75     |
| 4.4   | 3.7  | 6.3      | 5.4   | 8.5  | 1.5      |
| 4.5   | 4.25 | 5.75     | 5.5   | 8.8  | 1.2      |
| 4.6   | 4.8  | 5.2      | 5.6   | 9.05 | 0.95     |
| 4.7   | 5.4  | 4.6      | 5.7   | 9.25 | 0.75     |

**8. The determination of the  $P_H$  of urine.***Apparatus and reagents required.*

(1) A number of clean, dry test-tubes of thin clear glass and of uniform bore.  $\frac{5}{8}$  inch is a suitable external diameter.

(2) A comparator for holding the tubes. This is shown in figure 4.

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\* See page 18.



(3) A series of buffer solutions of known  $P_H$  prepared according to directions given above. It is convenient to have a series of these prepared and contained in bottles fitted with a rubber stopper, through which passes the stem of a 5 cc. pipette.

(4) Solutions of appropriate indicators (see page 23). Methyl red, brom-cresol-purple, brom-thymol blue cover the range of the majority of specimens of urine.

(5) A screen to cut out blue rays when using brom-cresol-purple (see page 23).

*Method.* To 5 cc. of the *filtered* urine add 5 drops of methyl red. If the mixture is red, the  $P_H$  is in the neighbourhood of 5. If it is yellow, the  $P_H$  is nearer 6. In the latter case treat another 5 cc. with 5 drops of brom-cresol-purple. A deep purple tint suggests that the  $P_H$  is higher than 6, in which case it may be necessary to use brom-thymol-blue. Having roughly obtained the range and the necessary indicator, place about 5 cc. of the specimen into two of the special tubes and place them in the holes marked 2 and 6. Into a tube in 4 place some distilled water.

Measure exactly 5 cc. of the urine into another tube, and to it add 5 drops of the indicator (measured with a Dreyer's dropping pipette, fig. 5). Mix by rotating the tube between the palms of the hands, and place the tube in the hole marked 3. Measure 5 cc. of one of the buffer solutions into a tube, add 5 drops of the indicator, mix, and place the tube in the hole marked 1. Hold the comparator to the source of light with the ground glass screen towards the observer and note the appearance opposite the slots  $x$  and  $y$ . It will then be ascertained whether the buffer chosen is acid or alkaline to the urine. In either case another buffer solution must be taken, the indicator added, and the tube placed in the hole 5, and the tubes examined again. This procedure must be repeated until two solutions are found of such a  $P_H$  that the colour as seen through  $y$  is intermediate between those seen through  $x$  and  $z$ , or that through  $y$  is identical with one of them. The  $P_H$  of the solutions finally employed should not differ by more than 0.1.

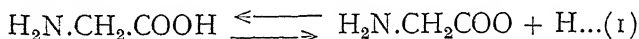
NOTE.—In measuring the indicator solutions it is essential to hold the dropping pipette vertical, to ensure the delivery of equal drops.

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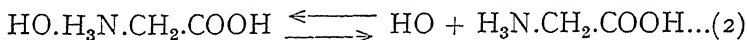
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### H. Ampholytes or amphoteric electrolytes.

These are substances which can function as acids by forming salts with bases, and also as bases by forming salts with acids. The amino acids, such as glycine, are examples. Glycine can form a sodium salt,  $\text{CH}_2\text{NH}_2\text{COONa}$ , and also a hydrochloride,  $\text{HCl.H}_2\text{N.CH}_2\text{COOH}$ . In strong acids it behaves as a base; in strong alkalies as an acid. In neutral solutions it is a feeble electrolyte, and is partially dissociated into H and a negative ion (anion).



and partially into OH and a kation.



If a strong acid, such as HCl, be added the dissociation (1) is decreased, in the same way as the dissociation of all weak acids is decreased by an increase in the hydrogen-ion concentration. On the other hand the number of kations formed is increased, since such a salt as glycine hydrochloride is freely dissociated.

If a strong base, like NaOH, be added the dissociation (2) is depressed, and there is an increase in the number of anions of the ampholyte, due to the free dissociation of the sodium salt that is formed.

For every ampholyte there is some particular concentration of hydrogen ions at which the dissociation (1) is equal to the dissociation (2). This is known as the "iso-electric point." Since the proteins are ampholytes, the conditions of a substance at its iso-electric point are of some interest. They are :

- (1) The sum of the anions and kations is at a minimum.
- (2) The concentrations of the anions and kations are equal.
- (3) If an ampholyte be added to a solution, whose [H] is greater than its iso-electric point, it functions as a base, and therefore decreases the [H] of the solution. If it be

added to a solution, whose  $[H]$  is less than its iso-electric point, it functions as an acid. If the  $(H)$  of a solution is not altered by addition of an ampholyte, then the  $(H)$  of the solution must be equal to the iso-electric point of the ampholyte.

(4) The solubility of an ampholyte is at a minimum at its iso-electric point. If a colloid the ampholyte flocks most readily at this point. (See Ex. 6.)

(5) At its iso-electric point, a colloid is electrically neutral.

The significance of the iso-electric points of various enzymes is discussed on p. 184. The iso-electric points of certain substances of physiological interest are shewn in Table II., p. 32.

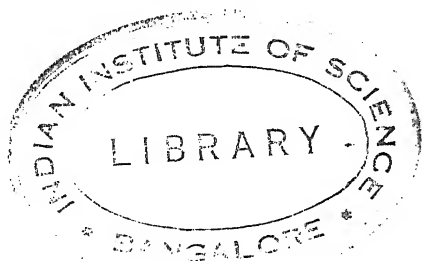


TABLE II.

| P <sub>H</sub> | RANGE OF PRINCIPAL INDICATORS | REACTIONS OF FLUIDS   | OPTIMUM REACTIONS   | ISOELECTRIC POINTS   | P <sub>H</sub> |
|----------------|-------------------------------|---|---|--|----------------|
| 10             |                               |   |   |  | 10             |
| 9              | PHENOL PHTHALEIN              |   |   |  | 9              |
| 8              | THYMOL BLUE                   | (Pancreatic juice (8.3)<br>Intestinal contents (8.3)  | → Trypsin on fibrin (8.0)<br>→ Erepsin (7.8)                                      |  | 8              |
| 7              | PHENOL BLUE                   | Blood (7.4)   |   | Histidine (7.2)  | 7              |
| 6              | BROM-THYMOL BLUE              | Human milk (7.1)<br>Pure water —<br>Saliva (6.9)<br>→ Cow's milk (6.7)  | — True neutrality —<br>(Maltase (6.7)<br>Ptyalin (6.7)<br>Trypsin on casein (6.7) | Alanine (6.7)<br>Oxyhaemoglobin (6.7)<br>Glycine (6.6)                         | 6              |
| 5              | BR-CRESOL PURPLE              | Urine (6.0)   |   |  | 5              |
| 4              | METHYL RED                    | Infants' gastric juice (5.0)  | Protease of Taka-diastase (5.1)   | Tyrosine (5.41)<br>Serum globulin (5.4)<br>Serum albumin (denaturated) (5.4)   | 4              |
| 3              | THYMOL BLUE                   | 0.0001 N. HCl (4.01)<br>0.001 N. acetic (3.87)<br>0.01 N. acetic (3.37)<br>0.001 N. HCl (3.01)<br>0.1 N. acetic acid (2.87)<br>N. acetic (2.37)<br>0.01 N. HCl (2.02) | → Invertase (4.5)   | Serum albumin (4.7)<br>Casein (4.6)<br>Gelatine (4.6)<br>Phenyl-alanine (4.48) | 3              |
| 2              |                               | Adult gastric juice (0.9 to 1.6)<br>0.1 N. HCl (1.08)   | Pepsin (1.4)  | Aspartic acid (2.9)  | 2              |
| 1              |                               |   |   |  | 1              |

## CHAPTER II.

### THE PROTEINS.

#### A. Definition.

Proteins are nitrogenous compounds found in the fluids and tissues of all living organisms. Chemically, they are composed of a number of amino acids (see p. 67), condensed together in a characteristic way so that the whole molecule is generally neither very acid nor very basic. Their chemical properties are dependent on the presence of these amino acids. Their physical properties are mainly due to the fact that they form colloidal solutions (p. 1). The percentage composition varies very considerably in different proteins. The following can be taken as a rough average :—

|       |   |    |           |
|-------|---|----|-----------|
| C     | = | 53 | per cent. |
| O     | = | 23 | „ „       |
| N     | = | 16 | „ „       |
| H     | = | 7  | „ „       |
| S     | = | 1  | „ „       |
| <hr/> |   |    |           |
| 100   |   |    |           |
| <hr/> |   |    |           |

#### B. Classification.

It is not possible at present to give a rational scheme, for we have not sufficient data of a chemical nature by means of which we can characterise the individual proteins.

The classification adopted here is based on physical and chemical properties, and closely follows the official classification of the American Physiological Society. Where the British Society uses a different name this is indicated by (B).

1. **Protamines.** Basic substances forming stable salts with mineral acids, and containing a high percentage of nitrogen. On hydrolysis they yield only a few of the amino acids, and these are mainly the bases. They occur in the heads of ripe spermatozoa and in ova.

2. **Histones.** Similar to the protamines, but less rich in nitrogen and the basic amino acids. They are, however, more basic than the majority of the proteins, and are precipitated by ammonia. They are found in unripe spermatozoa, the stroma of red blood corpuscles, and in lymphoid tissue.

3. **Albumins.** Soluble in distilled water and coagulated by boiling.

4. **Globulins.** Insoluble in distilled water, soluble in dilute salt solutions. Coagulated by boiling.

Groups 3 and 4 are sometimes known as "native" proteins.

5. **Glutelins.** Found in abundance in vegetables. Insoluble in neutral solvents, but soluble in acids and alkalis.

6. **Prolamines** [Gliadins (B)]. Also found in vegetables, but distinguished from the glutelins by their solubility in 75 per cent. alcohol.

7. **Albuminoids** [Scleroproteins (B)]. Found in the skeletal and connective tissues of animals. They are characterised by their insolubility in most reagents. Examples are keratin, elastin, and collagen (the anhydride of gelatine).

8. **Phospho-proteins.** Rich in phosphorus. They must be carefully distinguished from the nucleoproteins. Examples are the casein [caseinogen (B)] of milk and vitellin of egg yolk.

9. **Conjugated Proteins.** Proteins united to a non-protein group.

- (i.) *Chromoproteins.* Protein + pigment molecule, e.g. haemoglobin.
- (ii.) *Glycoproteins* [Glucoproteins (B)]. Protein + carbohydrate group, e.g. mucin.
- (iii.) *Nucleoproteins.* Protein + nucleic acid. These are probably indefinite salts of nucleic acid with proteins (see p. 60).

10. **Hydrolysed Proteins.** Formed by the action of acids, alkalies and certain enzymes on the native proteins.

(i.) *Metaproteins.* Soluble only in acids and alkalies.

(ii.) *Proteoses or albumoses.* Soluble in water, not coagulated by heat, precipitated by ammonium sulphate.

(iii.) *Peptones.* Like the albumoses, but not precipitated by ammonium sulphate.

(iv.) *Polypeptides.* Simple peptones, formed of mon-amino acids only.

### C. General Reactions.

For the following reactions use egg-white that has been well beaten with 6 times its volume of water or serum that has been diluted ten times with water.

(1.) The proteins give certain *colour reactions* (see pages 38 to 41).

(2.) They are *precipitated by the salts of the heavy metals*. The salts that are most used are lead acetate, mercuric chloride and nitrate, ferric chloride, copper sulphate, and zinc sulphate. The mechanism of the precipitation is somewhat complex, and probably varies for different salts and different concentrations. In a good many cases it seems to be due to the adsorption of the metallic kation by the negatively charged colloidal protein. For this reason the precipitation is best obtained when the reaction of the medium is somewhat alkaline, the protein then being negatively charged (see p. 11). Also the precipitate is often soluble in acid. It is often soluble in an excess of the metallic salt, probably because the charge on the protein becomes positive owing to the adsorption of the excess of positive ions.

9. Treat 3 cc. of the solution with a few drops of mercuric nitrate. A white precipitate is obtained. This will partially or completely dissolve in a saturated solution of sodium chloride, provided that the solution does not contain free acid. The solubility in sodium chloride is due to the fact that mercuric chloride is formed. This salt differs<sup>7</sup><sub>2</sub> from the nitrate in that it is only feebly dissociated.

10. Treat 3 cc. of the protein solution with ferric chloride, drop by drop. A precipitate is formed soluble in excess.

11. Treat 3 cc. of the protein solution with a solution of lead acetate or basic lead acetate. A white precipitate is formed.

(3.) *The proteins are precipitated by the so-called "alkaloidal reagents."* These include phosphotungstic, phosphomolybdic, ferrocyanic, tannic, picric, metaphosphoric, and sulphosalicylic acids, and Brücke's reagent (potassium-mercuric iodide).

It is possible that the precipitation is due to the adsorption of the complex negative ions by the positively charged colloidal protein. It is suggestive that the substances are only effective in acid solution, in which the proteins are positively charged. The precipitating action of these reagents on the peptones varies somewhat. As a rule they are not so readily precipitated as the albumins and globulins.

12. Treat 3 cc. of the solution with two or three drops of strong acetic acid and two drops of potassium ferrocyanide. A white precipitate is formed. Boil. The precipitate does not dissolve.

NOTES.—1. Primary proteoses are also precipitated by ferrocyanic acid, but the precipitate produced dissolves on warming and reappears on cooling (Ex. 55).

2. The precipitate and fluid often become coloured blue-green on boiling. This is due to a decomposition of the hydroferrocyanic acid on boiling it with certain organic substances, such as proteins.

13. Acidify some of the solution with hydrochloric acid and add a few drops of a freshly prepared solution of tannic acid, or of Almén's reagent. A white or brown precipitate is usually formed.

NOTE.—Almén's reagent consists of 4 gm. of tannic acid in 8 cc. of strong acetic acid and 190 cc. of 50 per cent. alcohol.

14. Treat 3 cc. with an equal volume of Esbach's solution. A yellowish precipitate is formed.

NOTE.—Esbach's solution is prepared by dissolving 10 grms. of picric acid and 10 grms. of citric acid in water and making the volume up to a litre. It is extensively used for the determination of albumin in urine (Ex. 421).



15. Acidify the solution with dilute hydrochloric acid and add a few drops of potassio-mercuric iodide (Brücke's reagent). A white precipitate is formed.

NOTE.—Brücke's reagent is prepared by dissolving 50 grms. of potassium iodide in 500 cc. of distilled water, saturating with mercuric iodide (120 grms.), and making up to 1 litre.

16. Acidify a few cc. of the solution with dilute hydrochloric acid and add a few cc. of a 2 per cent. solution of phosphotungstic acid. A white precipitate is produced.

17. To a few cc. of the solution add a drop or two of a freshly prepared 25 per cent. solution of metaphosphoric acid. A white precipitate is produced.

NOTE.—Metaphosphoric acid ( $\text{HPO}_3$ ) is used by Folin for removing proteins from blood and urine in certain quantitative methods. The solution must be freshly prepared, as on standing it slowly passes over into orthophosphoric acid ( $\text{H}_3\text{PO}_4$ ), which has no precipitating action on proteins.

18. To a few cc. of the solution add a small amount (a large "knife point") of sulphosalicylic acid, or a drop or two of a strong (20 per cent.) solution. A white precipitate is obtained.

NOTE.—The reagent is of considerable value for the detection of albumin in urine. (See Ex. 371.)

It can be prepared by dissolving 13 grm. salicylic acid in 20 grms.  $\text{H}_2\text{SO}_4$  by warming, and, after cooling, adding 67 cc. of water.

(4.) *The proteins are precipitated by strong alcohol.* The albumins and globulins are rapidly changed by alcohol at room temperature into modifications that are insoluble in water, salt solutions, dilute alkalis or acids. That is, they are coagulated.

The proteoses and peptones, the phosphoproteins, and gelatin are precipitated by alcohol, but the precipitate redissolves in water or dilute alkalis.

19. Place about 4 cc. of serum in a test-tube and cool to  $0^\circ\text{C}$ . by means of a freezing mixture. Fill the tube with strong alcohol that has previously been cooled to about  $8^\circ\text{C}$ ., and mix. A white precipitate of the proteins is formed. Filter at once and treat the precipitate with water. It dissolves.

20. Allow a few drops of serum to fall into about 10 cc. of strong alcohol at room temperature. A white precipitate is formed. Shake well and allow to stand for half an hour. Filter and treat the precipitate with water. It does not dissolve.

### D. Colour Reactions.

21. **The Xanthoproteic reaction.** To 3 cc. of the protein solution in a test-tube add about one cc. of strong nitric acid. A white precipitate is formed (see Ex. 40). Boil for a minute. The precipitate turns yellow and partly dissolves to give a yellow solution. Cool under the tap and add strong ammonia or soda till the reaction is alkaline. The yellow colour is turned to orange.

NOTES.—1. The essential features of the reaction are that a yellow colour is obtained when the solution is boiled with strong nitric acid, and that this yellow colour is intensified when the solution is made alkaline.

2. The precipitate is due to the formation of metaprotein by the action of nitric acid on albumins or globulins, this metaprotein being insoluble in strong mineral acids. It follows that proteoses and peptones, etc., do not give the precipitate with nitric acid.

3. The yellow colour is due to the formation of a nitro-compound of some aromatic substance, *i.e.* a substance containing the benzene ring.

4. The aromatic substances in the protein molecule that are responsible for the reaction are tyrosine, tryptophane and phenyl alanine.

5. Oleic acid, olive oil and most vegetable oils give a well-marked xanthoproteic reaction.

6. To test for traces of proteins proceed as follows: Boil with nitric acid and divide into two portions. Cool one portion and make it alkaline with ammonia. Compare the colour of the two portions. The alkaline tube will shew a faint yellow colour when only the merest trace of protein is present.

22. **Millon's reaction.** Treat 5 cc. of the protein solution with half its volume of Millon's reagent. A white precipitate is formed. Cautiously heat the mixture. The precipitate turns brick-red in colour, or disappears and leaves a red solution.

NOTES.—1. The essential feature of the reaction is the red colour on heating. The white precipitate in the cold is due to the action of the mercuric nitrate on the proteins. (See Ex. 9.)

2. A white precipitate is also obtained with solutions of urea. (See Exs. 341 and 342.)

3. Sulphates give a white precipitate of mercurous sulphate.

4. The reagent is prepared by dissolving one part by weight of mercury in twice its weight of concentrated nitric acid (Sp. gr. 1.42). The mixture is slightly warmed to the end. It is then treated with twice its bulk of distilled water, allowed to settle over-night, and filtered. It contains mercurous and mercuric nitrates, excess of nitric acid, and a small amount of nitrous acid.

5. The reaction should never be attempted with a strongly alkaline fluid, since the alkali will precipitate the yellow or black oxides of mercury.

6. If an excess of the reagent be employed the red colour is often discharged on boiling.

7. The reaction is given with all aromatic substances that contain a hydroxyl group attached to the benzene ring. Thus it is given by phenol, salicylic acid, and naphthol, but is not given by benzoic acid.

8. The aromatic substance derived from protein that is responsible for the reaction is tyrosine. 
$$\text{C}_6\text{H}_4 \begin{cases} \text{OH} \dots\dots\dots (1) \\ \text{CH}_2\text{CH.NH}_2\text{COOH} \end{cases} (4) \quad (\text{See Ex. 92.})$$

23. **The glyoxylic reaction.** (Hopkins and Cole.) Treat 2 or 3 cc. of the fluid with the same bulk of "reduced oxalic acid" ("glyoxylic reagent"). Mix and add an equal volume of concentrated sulphuric acid, pouring it down the side of the tube. A purple ring forms at the junction of the fluids. Mix the fluids by shaking the tubes gently from side to side. The purple colour spreads through the whole fluid.

NOTES.—1. The "glyoxylic reagent" is prepared by one of the following methods :—

A. Treat half a litre of saturated solution of oxalic acid with 40 grammes of 2 per cent. sodium amalgam in a tall cylinder. When all the hydrogen has been evolved the solution is filtered and diluted with twice its volume of distilled water. The solution now contains oxalic acid, sodium binoxalate, and glyoxylic acid ( $\text{COOH}.\text{CHO}$ ). It should be kept in a closed bottle containing a little chloroform.

B. In a flask place 10 grammes of powdered magnesium and just cover with distilled water. Slowly add 250 cc. of saturated oxalic acid, cooling under the tap at intervals. Filter off the insoluble magnesium oxalate, acidify with acetic acid, dilute to one litre with distilled water, and bottle as above.

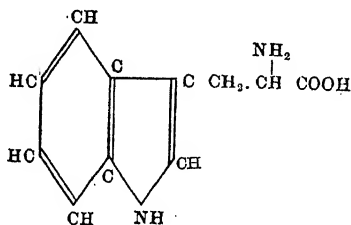
2. The reaction does not succeed in the presence of nitrates, chlorates, nitrites, or excess of chlorides.

3. The colour is not well seen if the protein is mixed with certain carbohydrates (e.g. cane-sugar), owing to the char produced by the strong sulphuric acid.

4. It is important to use pure sulphuric acid for this test. It sometimes fails owing to the presence of impurities in the acid employed. At the same time it must be admitted that a very minute trace of ferric chloride does sometimes increase the intensity of the colour.

5. In performing the test on a solid substance, like fibrin, or keratin, a small amount of the material should be heated with a few cc. of the reduced oxalic acid and an equal volume of strong sulphuric acid. The mixture is shaken, and as the protein dissolves in the strong acid both the fluid and the solid particles assume a purple colour.

6. The substance in the protein molecule that is responsible for the reaction is **tryptophane** (indol-amino-propionic-acid)  $C_{11}H_{12}N_2O_2$ .



7. A similar reaction can be obtained by using a very dilute (1:250) solution of formaldehyde containing a trace of an oxidising reagent like ferric chloride. The authors of the original reaction regarded this test (Rosenheim's) as being different from the glyoxylic test, though the whole question is still confused.

8. The author has shown that many substances, especially aldehydes, react with tryptophane to yield coloured products in the presence of strong HCl or  $H_2SO_4$ . Most of these reactions only succeed in the presence of an oxidising reagent, and are possibly due to a reaction with some oxidation product of tryptophane.

24. **The biuret reaction (Piotrowski's reaction).** Treat about 3 cc. of the solution with 1 cc. of 40 per cent. sodium hydroxide. Add a single drop of a 1 per cent. solution of copper sulphate. A violet or pink colour is produced.

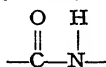
NOTES.—1. The reaction is of especial importance in testing for proteoses and peptones, which give a rose colour. It is generally stated that other proteins give a violet colour, but usually egg-albumin gives a distinct rose tint.

2. It is important to avoid an excess of copper sulphate, the blue copper colour obscuring the violet or rose tint.

3. The test cannot be applied in the presence of a large amount of magnesium sulphate, owing to the precipitation of magnesium hydroxide by the alkali.

4. If the solution contains much ammonium sulphate it must be treated with a large excess of strong sodium hydroxide, as directed in Ex. 57.

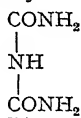
5. The reaction is given by nearly all substances containing two



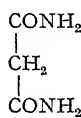
groups attached to one another, to the same nitrogen atom, or to the same carbon atom. Thus it is given by



Oxamide.

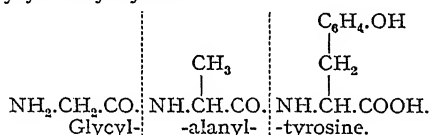


Biuret (Sec Ex. 345).



Malonamide.

The cause of the reaction with proteins is the presence of one or more groupings of the last type, formed by the condensation of the carboxylic group of an amino-acid with the amino group of another amino-acid. The linkage thus formed is known as the "peptide" linkage. Thus it would be given by the polypeptide, glycyl-alanyl-tyrosine

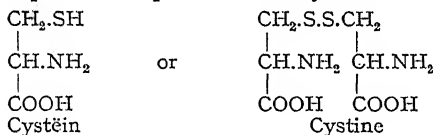


**25. The sulphur reaction.** Boil a little undiluted egg-white or serum with some 40 per cent. sodium hydroxide for two minutes, and then add a drop or two of lead acetate. The solution turns deep black.

NOTES.—1. This reaction is due to the fact that the sulphur of the protein is liberated as sodium sulphide when boiled with the strong alkali. The sulphide gives a black colour or precipitate of lead sulphide when the solution is subsequently treated with lead acetate.

2. The reaction does not succeed with caseinogen, peptones, and certain other proteins.

3. The sulphur in the protein is mainly combined as



**26. Molisch's reaction.** Treat 5 cc. of the diluted solution with three or four drops of a 1 per cent. solution of alpha-naphthol or of thymol in alcohol. Mix, and run about 5 cc. of concentrated sulphuric acid under the fluid. A violet ring is formed at the junction of the two liquids.

NOTES.—1. The reaction is due to the presence of a carbohydrate group (glucosamine) in the protein. This is converted by the acid to furfural, which condenses with the alpha-naphthol or the thymol to give the purple colour. (See notes to Exs. 110 and 114.)

2. A green ring is often seen in addition to the violet ring. This is due to the action of the sulphuric acid on the alpha-naphthol.

### E. The heat coagulation of albumins and globulins.

These proteins are placed in a class by themselves, because they exhibit most characteristically the phenomenon of *heat coagulation*.

A proper understanding of the conditions governing this phenomenon is so important that students are urged to study them attentively. The following matter should be re-read after the section on metaproteins has been studied.

When a solution of albumin or globulin is heated under certain conditions the protein separates in a form which is insoluble in water, dilute salt solutions, acids and alkalies. This is the phenomenon known as "heat coagulation." The term "coagulation" is used as an indication of an irreversible change and to distinguish the condition of the protein from that of "precipitation," in which re-solution can be brought about by a change of reaction, salt content, etc.

The two most important conditions affecting heat coagulation are reaction and salt content. It will be found later that albumins and globulins are readily converted into metaproteins by treatment with acids or alkalies, the conversion being much accelerated by a rise in temperature. The metaproteins are soluble in dilute acids or alkalies, but are insoluble in the neutral condition, *i.e.* in water or neutral salt solutions. An important fact about them is that if a *precipitate* of metaprotein is boiled it is "coagulated," that is, it will not redissolve in dilute acids or alkalies. The conversion of albumin or globulin into metaprotein is called "denaturation," and is a necessary antecedent of heat coagulation. This process is best regarded as an hydrolysis, which takes place at all reactions, but most rapidly in either acid or alkaline solutions. At boiling point the change is practically instantaneous, no matter what the reaction may be. Should the reaction be at the iso-electric point of the denaturised protein either the whole or

a considerable part of this is aggregated into flocks (see p. 11). At high temperatures these flocks are coagulated, that is, they do not redissolve on altering the reaction of the fluid.

The main effect of neutral salts is to aid the aggregation of the denaturated protein. It often happens that more than one denaturated protein is formed by heating a solution containing albumins and globulins. The iso-electric points of these proteins may differ, so that a certain proportion of the protein will remain non-coagulated at any given reaction. This seems to be true, even in the case of the denaturated protein formed from a pure protein. The addition of a neutral salt will tend to cause flocking of this "soluble" portion of the denaturated protein, and these flocks will be coagulated should the temperature be high enough. The non-coagulated dispersed protein carries an electric charge, which varies with the reaction, being positive in acid solutions and negative in alkaline solutions. We have seen that electro-positive colloids are flocked by negative ions, and electro-negative colloids by positive ions. Further, that this flocking power of the ions is much greater with di- and tri-valent ions than with mono-valent ions. It follows, therefore, that the ideal conditions for the maximal heat coagulation of an albumin or globulin are that the reaction should be at the iso-electric point of the denaturated protein formed, and that there should be present di- or tri-valent ions both positive and negative. These conditions are met by having the boiling solution very faintly acid to litmus and adding a trace of calcium chloride or magnesium sulphate. It is advisable to add the salt, to boil the solution, and then to change the reaction slowly by the addition of dilute sodium carbonate or 1 per cent. acetic acid, so that the final reaction is just acid to litmus. The exact point and procedure can only be determined by experience since it varies considerably with the concentration of the protein, etc.

For the following five exercises use serum that has been diluted with 10 volumes of distilled water.

27. Boil 5 cc. in a test-tube that has been previously rinsed with distilled water. The solution becomes opalescent, but usually no definite coagulum is formed. Cool the tube and add 1 per cent. acetic acid drop by drop. A precipitate of metaprotein is formed soluble in excess of acid.

NOTE.—The reaction of the mixture after boiling is distinctly alkaline to the iso-electric point of the denaturised proteins formed. These are precipitated by bringing the reaction of the solution to the iso-electric point by the addition of acetic acid, but are redissolved by an excess.

28. To 5 cc. add two drops of 1 per cent. acetic acid and boil. A white flocculent coagulum is formed. Cool the tube and add two or three drops of strong nitric or acetic acid. The coagulum does not dissolve.

NOTES.—1. The amount of acid added is such that, after boiling, the reaction is near to the iso-electric point of the denaturised proteins. These are therefore precipitated and then coagulated.

2. The addition of the strong acid is to ensure that the precipitate that appears on boiling does not consist of calcium or magnesium phosphate, which is soluble in dilute nitric acid. That such a phosphatic precipitate can be formed on boiling certain solutions is shown by the following experiment. Treat a solution of calcium chloride with sodium phosphate and then with excess of sodium carbonate. A precipitate of  $\text{Ca}_3(\text{PO}_4)_2$  appears. Add acetic acid drop by drop till the precipitate just dissolves owing to the formation of the acid phosphate. Boil the solution for half a minute. A white precipitate appears. Add a drop or two of nitric acid. The precipitate dissolves. The appearance of the precipitate of  $\text{Ca}_3(\text{PO}_4)_2$  on boiling is due to the alteration of reaction as the  $\text{CO}_2$  is evolved.

29. Treat 5 cc. of the solution with 0.4 per cent. hydrochloric acid, drop by drop, until the precipitate obtained by the first drop or two has redissolved (about five drops are usually necessary). Boil. The solution remains clear. Cool the tube and add 2 per cent. sodium carbonate, drop by drop. A precipitate of metaprotein is formed which redissolves in excess.

NOTE.—The precipitate that first forms consists of globulin (see Ex. 32). On adding enough HCl to redissolve this the reaction is such that it is acid to the iso-electric point of the denaturised proteins. These are precipitated by an alkali and redissolve in an excess.

30. To 5 cc. add two drops of 2 per cent. sodium carbonate and boil. The solution remains quite clear. Cool the tube and add 1 per cent. acetic acid, drop by drop. A precipitate of metaprotein is formed, soluble in excess.



NOTE.—The results in this exercise are similar to those obtained in Ex. 27, except that the increased alkalinity prevents the formation of the opalescence obtained in the absence of added alkali.

31. To 5 cc. add a drop of 2 per cent. calcium chloride and boil. A considerable coagulum is obtained.

NOTE.—Though the solution is alkaline to the iso-electric point, the divalent positive calcium ion precipitates a certain proportion of the negative colloidal protein.

### F. The properties of albumins and globulins.

**Globulins** are generally insoluble in distilled water, but soluble in dilute acids and alkalies, and in weak solutions of neutral salts.

A neutral solution in a dilute salt is coagulated on boiling.

A solution in dilute acid or alkali is converted into a solution of metaprotein on boiling.

If the globulin be dissolved in a minimum amount of a neutral salt solution and the solution be diluted with several volumes of distilled water, the globulin is partially precipitated, for a certain *concentration* of salt is necessary to keep the globulin in solution. If the globulin be dissolved in dilute acid or alkali, there is no precipitation on dilution.

The globulins are completely precipitated by full saturation with magnesium sulphate or by half-saturation with ammonium sulphate, *i.e.* by treatment of the solution with an equal volume of a saturated solution of ammonium sulphate.

**Albumins** are soluble in distilled water, dilute salt solutions, dilute acids and alkalies.

A neutral solution in water or salt is coagulated on boiling.

A solution in dilute acid or alkali is converted to a solution of metaprotein on boiling.

Solutions of albumins are not precipitated by saturation with magnesium sulphate nor by half-saturation with

ammonium sulphate if the reaction of the solution be neutral or alkaline.

They are partially precipitated by solutions of these substances in the presence of acid.

They are completely precipitated by full saturation with ammonium sulphate from a neutral, acid, or alkaline solution.

The solubility in water of the globulins of blood serum is apparently modified by the presence of certain "lipines" (see p. 153). If the serum globulins be precipitated by half saturation with ammonium sulphate, the precipitate dissolved in water, as described in Ex. 36, and the solution thoroughly dialysed, it will be found that only a portion of the protein is precipitated by the dialysis. The fraction that remains soluble in water has been called "pseudo-globulin" to distinguish it from the water-insoluble fraction or "eu-globulin." It was formerly believed that "pseudo-globulin" was an albumin and that it was impossible to separate the globulins from the albumins by half saturation with ammonium sulphate. Recent work by Hartley on the globulins of serum and by Raistrick on those of milk have demonstrated, however, that the distribution of nitrogen as mon-amino acids and as bases is practically the same for the two globulin fractions, which differ appreciably from the albumin fraction in this respect. It is probable that the insolubility of the "eu-globulin" in water is due to its association with lipid.

32. Dilute 5 cc. of serum with 50 cc. of distilled water. A faint cloud of serum globulin is formed. Cautiously add 0.4 per cent. hydrochloric acid or 1 per cent. acetic acid until the cloud has reached its maximum density. Divide into two portions A and B. To A add a couple of drops of a saturated solution of ammonium sulphate. The solution becomes quite clear. To B add a couple of drops of strong acid. The cloud disappears.

NOTE.—The globulin of the serum is held in solution both by salts and alkalis. Dilution alone produces a very small precipitate, but if the solution be now treated with just sufficient acid to neutralise the alkali, a much larger fraction of the globulin is thrown down. This globulin is soluble in dilute neutral salts, or in an excess of acid.

33. Prepare a suspension of globulin by the following method. To 15 cc. of serum in a beaker add 2 cc. (about 30 drops) of 1 per cent. acetic acid and 100 cc. distilled water. Stir and allow the mixture to stand for about 20 minutes. A precipitate of globulin settles down. Very carefully pour off the supernatant fluid and divide the suspended globulin into two equal portions in clean test-tubes. With these perform the two following exercises.

34. Add a 5 per cent. solution of sodium chloride, drop by drop, till the globulin has just dissolved. It is not easy to get a crystal clear solution, probably owing to the presence of a trace of some other protein (nucleo-protein). Divide the solution into three portions, (a), (b) and (c).

(a) Boil. The protein is coagulated.

(b) Dilute with about five volumes of distilled water. The globulin is partially reprecipitated.

(c) Treat with an equal volume of saturated ammonium sulphate solution. The globulin is reprecipitated.

35. Add 0.4 per cent. HCl, drop by drop, till the globulin has *just* dissolved. Divide the solution into three portions, (d), (e) and (f).

(d) Add 2 per cent. sodium carbonate solution till the globulin is partially reprecipitated (one or two drops only are necessary). Now add a few drops of 5 per cent. sodium chloride. The precipitate of globulin redissolves.

(e) Boil the solution. The protein is not coagulated. Cool under the tap and add enough 2 per cent. sodium carbonate to precipitate the metaprotein that has been formed by boiling. Now add a few drops of 5 per cent. sodium chloride. The precipitate of metaprotein does not dissolve.

(f) Dilute with about five volumes of distilled water. The globulin is not thrown out of solution.

36. Mix about 10 cc. of undiluted serum with an exactly equal quantity of a saturated solution of ammonium sulphate. A thick white precipitate is formed consisting of the whole of the globulin. Filter through a dry filter paper into a dry test-tube. Label the filtrate A. Scrape the precipitate off the paper and treat it with distilled water. The precipitate dissolves, the ammonium sulphate adhering to it forming a dilute salt solution which allows the globulin to go into solution. Boil a portion of this solution. A heat-coagulum is formed.

37. Filtrate A contains serum-albumin in the presence of half-saturated ammonium sulphate. Apply the following tests:

(a) Boil a portion. A heat-coagulum is formed.

- (b) To another add one drop of strong acetic acid. A white precipitate of serum-albumin is formed.
- (c) Grind the remainder in a mortar with solid ammonium sulphate, till the fluid is saturated. A white precipitate of serum-albumin is formed. Filter off the precipitate and test the filtrate for proteins either by boiling or by the glyoxylic or xanthoproteic reactions. Proteins are absent, showing that all the proteins of serum are precipitated by complete saturation with ammonium sulphate.

NOTE.—A certain test for albumin in a solution is to half-saturate it with ammonium sulphate, filter off any precipitate that may be present and boil the filtrate. A heat-coagulum indicates albumin.

38. Serum has been dialysed in collodion sacs (see p. 2) for 24 hours against distilled water in a tall cylinder. Note the heavy precipitate of serum-globulin that has fallen to the bottom of the sac. Pipette off some of the clear fluid and add an equal volume of saturated ammonium sulphate. A precipitate of "pseudo-globulin" (see note on p. 46) is obtained. Now pipette off some of the deposit, add about two volumes of distilled water, and divide into three portions, A, B, and C. To A add a couple of drops of saturated ammonium sulphate. To B add a drop of dilute soda. To C add a drop or two of dilute HCl. The globulin dissolves in each case.

39. Dilute 3 cc. of serum with about five times its volume of tap water, add a drop of 2 per cent. calcium chloride, and boil the mixture in a boiling tube. Add one drop of 1 per cent. acetic acid and boil again. Continue this procedure until a definite coagulum has formed, and the fluid between the flocks appears to be clear when examined in a thin layer. Filter. The filtrate should run through the paper rapidly and be crystal clear. If it filters slowly or comes through opalescent, repeat the experiment until the desired result is obtained. Test the filtrate for proteins by Millon's and the xanthoproteic tests. Only insignificant traces should be found.

NOTE.—This is the method usually adopted for removing albumins and globulins from solution, but it must be noted that it is almost impossible to remove the last traces by this procedure. If it is necessary to do so, colloidal iron (see Ex. 310), metaphosphoric acid (see Ex. 17), or some other reagent must be employed. The objection to the use of such reagents is that they are apt to precipitate the proteoses, peptones, etc.

40. **The action of mineral acids on albumins and globulins. (Heller's test.)** Place a few cc. of strong nitric acid in a narrow test-tube. By means of a pipette add an equal volume of dilute serum or egg-white, inclining the tube during the addition so that the protein solution is "layered" on the surface of the acid. A white ring appears at or immediately above the junction of the two fluids.

NOTE.—This is one of the most important tests for albumins in urine. The reaction is also given by  $\text{HCl}$  and  $\text{H}_2\text{SO}_4$ , but not so readily as by  $\text{HNO}_3$ . The primary proteoses also give a precipitate but this is soluble on warming.

### G. The chemistry of egg-white.

41. In egg-white which has been well beaten with a whisk (to break up the containing membranes), and diluted with four times its volume of distilled water, note a precipitate of ovo-mucin and globulin. Perform the following tests:

- (a) Take the reaction to litmus. It is alkaline.
- (b) Cautiously neutralise with dilute acetic acid. A slight increase in the precipitate of ovo-mucin and globulin is noticed. Remove this by filtration if necessary, and with the filtrate perform the following reactions:
- (c) Boil a portion. A coagulum is formed, indicating the presence of either a globulin or an albumin.
- (d) Make another portion very faintly alkaline by the addition of a drop or two of 2 per cent.  $\text{Na}_2\text{CO}_3$ . Now add an equal bulk of saturated  $(\text{NH}_4)_2\text{SO}_4$ . A slight precipitate of globulin or albumin is formed. Filter this off, and boil a portion of the filtrate with a drop of 1 per cent. acetic acid. A coagulum of albumin is formed. Saturate the remainder of this filtrate with ammonium sulphate by grinding with the solid in a mortar. A precipitate of albumin is formed.
- (e) Completely remove the globulin and albumin by boiling. Filter and apply Millon's or the xanthoproteic protein test to the filtrate. Protein is found in small quantities.

This protein is known as ovo-mucoid. It is not coagulated by boiling, nor precipitated by acetic acid. It is precipitated by saturation with ammonium sulphate, and also by strong alcohol.

**42. The crystallisation of egg-albumin.** (Hopkins' method.) Separate the white from a number of new-laid eggs, taking care not to allow any of the yolk to mix with the white. Measure the egg-white and churn it up with an exactly equal volume of a neutral fully-saturated solution of ammonium sulphate by means of a whisk, adding the sulphate in portions and mixing thoroughly after every addition. Notice the strong smell of ammonia that is evolved. Filter the mixture through a large pleated filter-paper. Measure the filtrate. Take 100 cc. of it and cautiously treat it with 10 per cent. acetic acid from a burette, noting the original level of the acid in the burette. Add the acid a drop or two at a time, shaking gently the whole time, until the precipitate produced at each addition no longer dissolves on shaking, and the whole mixture is rather opalescent. This point is usually somewhat difficult to determine, owing to the large number of air-bubbles that become suspended in the fluid and closely resemble a fine precipitate. When you are satisfied that a permanent precipitate has been produced, run in 1 cc. of the acid in addition to the amount already added. A heavy white precipitate is thus produced. Note the amount of acid that has been used for the portion of 100 cc., and treat the remainder of the filtrate with a corresponding amount of acid. Mix the two portions thoroughly and allow to stand overnight. Note that the precipitate has increased somewhat in amount. Mount a drop of the suspension on a slide, cover with a slip, but do not press. Examine under the high power of the microscope, and note the aggregation of very fine needles.

The albumin can be recrystallised by filtering, dissolving in as small an amount of water as possible, filtering again, and cautiously adding to the filtrate saturated ammonium sulphate till a faint permanent precipitate is produced. If the mixture be allowed to stand for some hours the albumin will separate out as fine needles.

**NOTES.—1.** For the experiment to succeed it is absolutely essential that all the eggs employed be perfectly fresh. One rather stale egg may interfere with the crystallisation of a large number of fresh eggs

2. It is important to add exactly the amount of acetic acid mentioned, that is, one per mille above the amount required to give a faint permanent precipitate.

3. The same method can be employed for the crystallisation of serum-albumin from the perfectly fresh serum of a horse, ass or mule.

### H. The Metaproteins.

The metaproteins are derived from the albumins and globulins by hydrolysis. This can be effected rapidly by dilute acids and alkalis at temperatures over  $60^{\circ}\text{C}$ . (see Exs. 29 and 30): more slowly at body temperature. They are formed immediately by the action of strong mineral acids at room temperature. They are insoluble in water, *strong* mineral acids, and all solutions of neutral salts, but are soluble in dilute acids or alkalis in the absence of any large amount of neutral salts. They are not thrown out of solution (in acid or alkali) by boiling. But if such a solution be neutralised or precipitated by the addition of an excess of a neutral salt, the suspended metaprotein is coagulated on boiling, so that it will no longer dissolve in acid or alkali.

**Preparation.** Egg-white or serum is diluted with ten times its volume of either 0.4 per cent. hydrochloric acid or 0.1 per cent. sodium hydroxide and the mixture placed in a water bath or incubator at  $40^{\circ}\text{C}$ . for about twenty-four hours. The albumins and globulins are hydrolysed to metaprotein.

43. Neutralise about 25 cc. with 2 per cent. sodium carbonate, or 0.4 per cent. HCl, depending on the original reaction of the fluid. A bulky precipitate of metaprotein forms. The acid or alkali should be added until the maximum amount of precipitate is produced. The reaction then will probably be very slightly acid to litmus. Filter. The filtrate generally comes through very slowly. When as much as possible of the fluid has been removed in this way transfer the fluid on the paper to a small beaker, open the paper, and add the precipitate to the fluid that has been poured off, dilute with a little water, and divide the suspension into six equal portions and with them perform the following six exercises.

44. Add some 0.4 per cent. HCl. The precipitate dissolves. Neutralise with sodium carbonate: the precipitate reappears.

45. Add concentrated HCl drop by drop. The precipitate dissolves with the first drop, but generally reappears when an excess is added (see Ex. 21, note 2, and Ex. 40).

46. Dissolve in a little 0.4 per cent. HCl. Boil the solution: a coagulum is not formed. Cool under the tap and neutralise with 0.2 per cent.  $\text{Na}_2\text{CO}_3$ . A precipitate is formed which is soluble in an excess of the alkali.

47. Boil. Cool, and add some 0.4 per cent. HCl. The precipitate does not dissolve, *i.e.* metaprotein is coagulated when boiled in suspension.

48. Add a saturated solution of ammonium sulphate drop by drop. The precipitate does not dissolve in any dilution of the salt. The insolubility in dilute solutions of neutral salts is an important distinction between metaproteins and globulins (see Ex. 32 and 34).

49. Dissolve in a little 0.4 per cent. HCl. Treat the solution with an equal volume of saturated ammonium sulphate solution. The protein is precipitated.

### I. The Albumoses or Proteoses and Peptones.

These hydrolysed proteins are obtained by the further action of acids or alkalis on globulins, albumins and metaproteins. They are best formed by the action of pepsin and hydrochloric acid on these proteins. Peptone is the end product of gastric digestion.

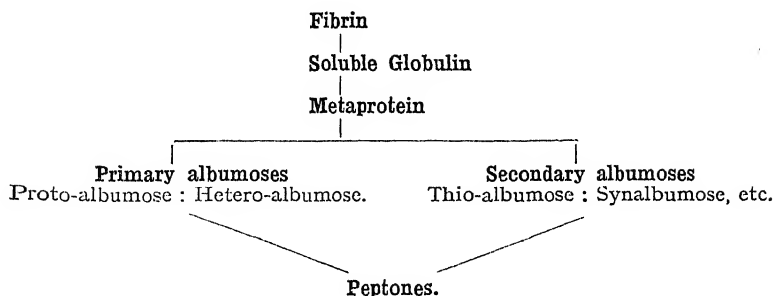
They are prepared on a commercial scale and sold as—

- (i.) Witte's peptone, which is prepared from fibrin and consists of a mixture of albumoses and peptone.
- (ii.) Savory and Moore's peptone, which is prepared from meat, and only contains traces of albumoses.

The following scheme indicates the successive steps



in the digestion of fibrin by pepsin and 0.2 per cent. hydrochloric acid :—



The following scheme shews the method adopted for the isolation of certain of the albumoses :—

**Neutral Witte's peptone**, treated with equal volume of saturated ammonium sulphate solution.

|   |  |  |  |                                     |  |
|---|--|--|--|-------------------------------------|--|
| <i>Precipitate</i> : dissolved in water.<br>Treated with 2 volumes of strong alcohol. |  | <i>Filtrate</i> , treated with half its volume of saturated ammonium sulphate. |  |                                     |  |
| <i>Ppt.</i><br><b>Hetero-albumose.</b>  | <i>Filtrate.</i><br><b>Proto-albumose.</b> | <i>Ppt.</i> dissolved in water.<br>Treated with 2 volumes of alcohol.          | <i>Filtrate</i> Saturated with ammonium sulphate.                            |                                     |  |
|   |  | <i>Ppt.</i> Thio-albumose.   | <i>Ppt.</i> dissolved in water.<br>Treated with 2 volumes of strong alcohol. | <i>Filtrate.</i><br><b>Peptones</b> |  |
|   |  |  | <i>Ppt.</i> Neglect.   |                                     |  |
|   |  |  | <i>Filtrate.</i> Treated with $\frac{2}{3}$ vols. of alcohol.                |                                     |  |
|   |  |  | <i>Ppt.</i> Synalbumose.   |                                     |  |

The **primary albumoses** are soluble in water, dilute acids, alkalis and salt solutions. Their solutions are not coagulated on heating. They are precipitated by half-saturation with ammonium sulphate. They give a pre-

precipitate, that disappears on warming and reappears on cooling, either with nitric acid or potassium ferrocyanide and acetic acid. They also give a precipitate in the cold with copper sulphate.

They give all the ordinary protein colour reactions, with the exception of Molisch's.

The **secondary albumoses** have somewhat similar properties to those of the primary albumoses: but they are not precipitated by nitric acid, ferrocyanic acid, or copper sulphate.

They require more than half-saturation with ammonium sulphate to precipitate them, but are completely precipitated by full saturation. Thio-albumose gives all the protein colour reactions and is particularly rich in sulphur (hence its name).

Synalbumose gives the protein reactions, with the exception of the glyoxylic test.

The **peptones** are very soluble proteins of rather a low molecular weight, so that they slowly diffuse through parchment membranes. They are the only proteins not precipitated by full saturation with ammonium sulphate. They fail to give precipitates with Esbach's and Brücke's reagents or ferrocyanic acid, but are precipitated by other protein precipitants, as tannic acid, phosphotungstic acid and lead acetate.

For the following reactions make a 5 per cent. solution of "Witte's peptone" in hot water, just acidify with acetic acid and filter from a small amount of insoluble material (nuclein?). The solution contains all the albumoses and peptones.

50. Dilute a small amount with three or four times its bulk of water, and to portions of this apply the usual colour reactions for protein. They are all obtained. Note, in particular, that the biuret test gives a rose colour.

51. Boil the solution with a trace of acetic acid: it does not form a coagulum.

52. Add a little tannic acid : a white précipitate is formed.

53. Add a little Esbach's or Brücke's solution : a yellow or white precipitate is formed.

54. Add a little lead acetate solution : a white precipitate is formed.

55. To 10 cc. of the 5 per cent. solution in a small beaker add 10 cc. of a saturated solution of ammonium sulphate. A white precipitate of the primary albumoses is formed. Stir the mixture vigorously for a short time with a glass rod that has one end covered with a small piece of rubber tubing : allow to stand for a few minutes. The precipitate will usually gather together and can be almost completely collected as a gummy mass on the end of the rod. Transfer it to about 5 cc. of hot water. The precipitate dissolves. Cool the solution and divide it into three portions.

(a) Add a drop of strong acetic acid and two drops of potassium ferrocyanide. A white precipitate is formed, which disappears on heating and reappears on cooling.

(b) To another portion add a few drops of strong nitric acid. A white precipitate is formed, which disappears on heating and reappears on cooling.

(c) To the third portion add a drop of copper sulphate solution. A white precipitate is formed.

56. The fluid from which the main mass of primary albumoses has been removed is filtered and treated in a beaker with a single drop of sulphuric acid, and then with ammonium sulphate that has been finely powdered in a mortar. The mixture is stirred vigorously till the fluid is saturated with the salt. A flocculent precipitate of the secondary albumoses (deutero-albumoses) is formed. Collect this on the rod as before, dissolve in a little water, divide the solution into three parts, and repeat the three tests already performed with the primary albumoses. A precipitate is not formed by any of the reagents.

57. The fluid from which the secondary albumoses have been removed contains peptone. Filter it, and treat a portion of the

filtrate with twice its volume of 40 per cent. sodium hydroxide and a drop of 1 per cent. copper sulphate. A pink colour appears, due to the presence of peptone.

**Important Note.**—This large excess of strong NaOH **must** be added in order to decompose the  $(\text{NH}_4)_2\text{SO}_4$  with which the solution is saturated. The characteristic rose colour is only obtained when the alkalinity is due to NaOH, ammonia being quite inefficient.

5 cc. of saturated  $(\text{NH}_4)_2\text{SO}_4$  solution contains about 3.75 grms. of the salt. This requires 2.27 grms. of NaOH. 10 cc. of 40 per cent. NaOH, containing 4 grms. of NaOH, is thus sufficient.

58. Evaporate a small portion of the original fluid to complete dryness, finishing the process on a water bath in order to prevent charring. Rub up the residue with successive small quantities of strong alcohol (95 per cent.). Add the extracts together, filter and evaporate them to dryness on a water bath. Dissolve the residue from this evaporation in a little water and test for proteins by the various colour tests. Only insignificant traces are present, showing that albumoses and peptones are insoluble in strong alcohol.

**NOTE.**—It is frequently desirable to remove all proteins from a solution before testing for certain substances, *e.g.* sugars, bile-salts, urea, etc. In the case of albumoses and peptones this can only be effected by the method described above, advantage being taken of the solubility of sugars, etc., in alcohol, and the insolubility of all proteins in the same. The aqueous solution prepared in this way will be spoken of as "an alcoholic extract."

**Peptones.** Use a 2 per cent. solution of Savory and Moore's peptone, which is usually free from albumoses.

59. Apply the usual colour reactions for proteins. They are all obtained.

**NOTE.**—The glyoxylic reaction may not be very intense, owing to the presence of chlorides in the preparation. Pure peptone, when freed from chloride by appropriate means, gives a very good glyoxylic reaction.

60. Add a drop or two of strong acetic acid and a drop of potassium ferrocyanide. No precipitate is produced, showing that the primary albumoses are absent.

61. Add a little Esbach's or Brücke's solution. A very slight or no precipitate is formed, if the solution be free from albumoses.

62. Saturate a portion with ammonium sulphate. No precipitate, or only a slight one, is produced, showing that albumoses are absent.

63. Treat 5 cc. of the filtrate from Ex. 62 with two volumes of 40 per cent. NaOH and a drop of copper sulphate. A pink colour is formed.

64. Add a few drops of a solution of tannic acid. A white precipitate is formed.

65. Add a few drops of a solution of lead acetate. A white precipitate is formed.

### J. The Gluco-proteins.

These bodies are conjugated proteins, the protein being united to a carbohydrate group.

They consist of the mucins and mucinoids or mucoids. The mucins are found in connective tissue and are secreted by certain of the salivary glands and various parts of the alimentary canal, notably the large intestine. Their solutions are viscous. They are soluble in dilute alkalis and are precipitated from solution by acetic acid, the precipitate being insoluble in excess of acetic acid. They are also soluble in 0.1 per cent. hydrochloric acid. On hydrolysis with acids the sugar group is split off and will reduce Fehling's solution.

The mucoids are not so viscous and not so readily precipitated by acetic acid, the precipitate being soluble in excess. They are found in ovarian cysts and in white of egg [see Ex. 41 (e)].

**Preparation of Mucin.** Mince the submaxillary gland of an ox, grind with sand and add 0.1 per cent. NaOH (1 litre to 50 grams of the moist gland). Shake well in a large bottle from time to time and leave for about half an hour. Strain through muslin and filter through coarse filter-paper. (This crude solution should not be prepared too long before use, as mucin loses its characteristic properties if left standing with alkalis.)

66. Add acetic acid drop by drop. A stringy precipitate is formed, insoluble in excess of the acid.

67. Remove the precipitate on a glass rod, wash with water, and apply the usual colour reactions for proteins, *e.g.* xanthoproteic, glyoxylic, and Millon's. They are all given by mucin.

68. Treat some of the precipitate with 0.1 per cent. HCl. The mucin dissolves.

69. Treat some of the precipitate with 2 per cent.  $\text{Na}_2\text{CO}_3$ . The mucin dissolves.

### K. The reactions of certain Albuminoids.

**Gelatin** is found in the body in the form of its anhydride, collagen. This occurs in white fibrous tissue and in the organic substance of bones, and can be converted into gelatin by boiling with a dilute acid. Dried gelatin swells in cold water, but is quite insoluble in it. On warming, a more or less viscid solution is obtained, which solidifies to a jelly on cooling provided the concentration be greater than 1 per cent. This process is reversible on warming and cooling. It is precipitated by half-saturation with ammonium sulphate, by tannic acid, phosphotungstic acid, Esbach's and Brücke's reagents, but not by normal lead acetate. On complete hydrolysis it yields a high percentage of its nitrogen in the form of glycine, but only traces in the form of the aromatic amino-acids, tyrosine, or tryptophane, and none as the sulphur-containing compound, cystine. Therefore its solutions fail to give the glyoxylic, Millon's and sulphur colour tests for proteins, and only give a slight xanthoproteic test, which is due, either to an impurity or to a small amount of phenyl-alanine.

70. Break gelatin up into small pieces and add a small amount of cold water. The gelatin does not dissolve. Immerse the test-tube in a beaker of boiling water and leave it for a short time. The gelatin dissolves. Cool the tube under the tap: the gelatin sets to a jelly. Perform the following tests with an approximately 1 per cent. solution of gelatin:

- (a) Xanthoproteic reaction: slight.
- (b) Millon's reaction: very slight, showing absence of tyrosine from gelatin molecule. (See notes to Ex. 22.)
- (c) Glyoxylic reaction: not obtained, showing absence of tryptophane. (Ex. 23.)

- (d) Biuret reaction : violet colour.
- (e) Sulphur reaction : not obtained, showing absence of cystine.  
(Ex. 25.)
- (f) Add acetic acid : no precipitate.
- (g) Add acetic acid and potassium ferrocyanide : very slight or no precipitate.
- (h) Add tannic acid : white precipitate.
- (i) Add lead acetate : very slight or no precipitate.
- (j) Half saturate with ammonium sulphate. The whole of the gelatin is precipitated, as shown by a negative biuret test in the filtrate (distinction from peptones).
- (k) Add Esbach's or Brücke's solution : yellow or white precipitate (distinction from peptones).

**Keratin.** An insoluble body found in the hair, skin, nails, and horns. Remarkable for the high percentage of cystine it yields on acid hydrolysis.

71. Perform the following tests by using horn shavings, or hair. Note insolubility in hot or cold water, dilute acids. and dilute alkalis.

- (a) Xanthoproteic reaction : well marked.
- (b) Millon's reaction : well marked.
- (c) Glyoxylic reaction : well marked.
- (d) Biuret reaction : not obtained, owing to insolubility.
- (e) Sulphur reaction : well marked.

## CHAPTER III.

### THE NUCLEOPROTEINS, NUCLEINS AND NUCLEIC ACIDS.

Nucleic acid is a complicated organic acid containing phosphorus, which is found widely distributed in animal and vegetable tissues. It is a special constituent of the nuclei and is therefore most abundant in cellular organs, such as the thymus, the pancreas, the testis, and the lymphatic glands.

Nucleic acid forms salt-like combinations with proteins, the amount and nature of the protein combining with the nucleic acid varying considerably. Such combinations are known as *nucleoproteins*. They are soluble in water and dilute salt solutions. They show acidic properties, being soluble in alkalies and precipitated by dilute acids. They dissolve to form an opalescent solution in excess of strong acetic acid. (Distinction from mucin.)

A rather special form of nucleoprotein is *nucleohistone*, in which the nucleic acid is combined with the basic protein, histone. It has similar physical properties to those of the other nucleoproteins, but is precipitated as a calcium compound by 0.2 per cent. calcium chloride solution.

On digesting nucleoprotein with pepsin and hydrochloric acid, the greater part of the protein is removed as peptone, but a certain amount is still left combined with the nucleic acid. This compound is known as *nuclein*. It is insoluble in water and dilute salt solutions, but is soluble in dilute alkalies.

By hydrolysis of nuclein by pancreatic juice or better by dilute alkalies, the remainder of the protein is removed, and there is obtained *nucleic acid*.

Nucleic acid is not hydrolysed by trypsin, but it is



broken down by a variety of ferments found in the tissues. The final products of hydrolysis of thymus nucleic acid are

Phosphoric acid.

Purine bases, adenine, and guanine.

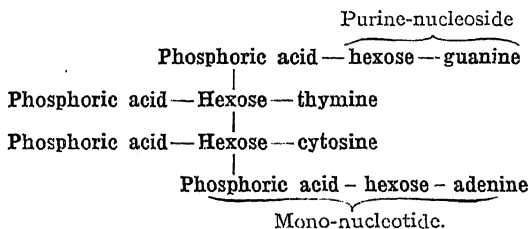
Pyrimidine bases, thymine, and cytosine.

An unknown hexose sugar.

Yeast nucleic acid differs only in yielding uracil instead of thymine and a pentose sugar (*d*-ribose) instead of the hexose. As to the composition of the nucleic acids, it has been established that they consist of certain groups called *nucleotides*, which can be liberated by the action of enzymes found in the tissues, and called nucleotidases. There are apparently four nucleotides to the molecule of nucleic acid. The nucleotides consist of phosphoric acid-sugar-base, the latter being either a purine base or a pyrimidine base. By the action of an enzyme, called phospho-nuclease, on the mononucleotides the phosphoric acid is split off, leaving the carbohydrate attached to the purine or pyrimidine base. These compounds are known as purine—or pyrimidine—*nucleosides*.

The nucleotides can, however, be attacked by another enzyme, purine—or pyrimidine—nuclease, which splits off the base from the phosphoric acid-sugar complex.

The following scheme, suggested by Levene and Jacobs, may represent the structure of thymus-nucleic-acid.

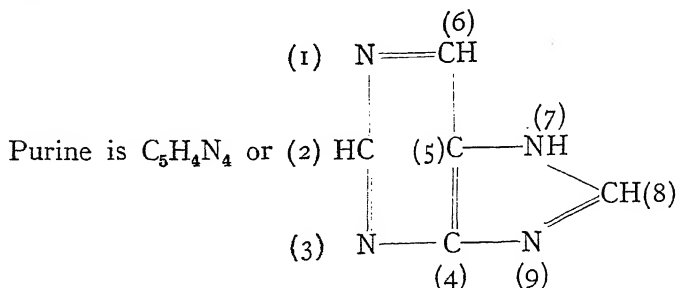


For further information on the subject the student is referred to the valuable monograph by W. Jones.\*

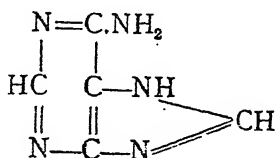
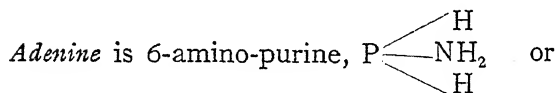
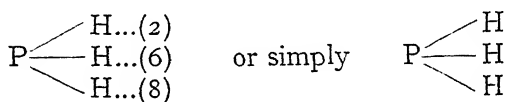
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\* *Nucleic Acids*, by Walter Jones. (Longmans, Green & Co., London, 1914.)

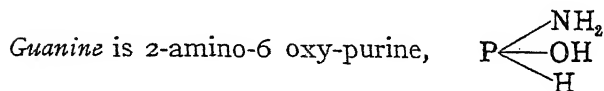
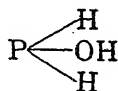
The *purine bases* are of especial interest in connection with the origin of uric acid.



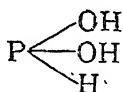
The figures in brackets are used for indicating the position of substitution groups. The chief purines of physiological interest have either the (2), (6), or (8) H atoms replaced, so we can write purine as



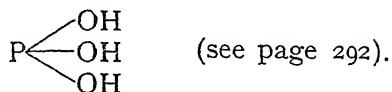
By the action of a deaminising enzyme, adenase, it is converted to *hypoxanthine*, or 6-oxy-purine,



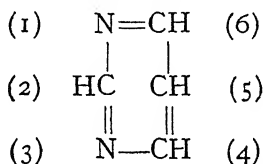
It is converted by guanase into di-oxy-purine, or *xanthine*,



Hypoxanthine, or xanthine, are oxidised by xanthin oxydase into 2, 6, 8 tri-oxy-purine, or *uric acid*



The *pyrimidine bases* are less complicated than the purine bases. The pyrimidine ring is



Uracil is 2-6-di-oxy-pyrimidine.

Thymine is 2-6-di-oxy-5-methyl pyrimidine, or 5-methyl uracil.

Cytosine is 6-amino-2-oxy-pyrimidine.

Practically nothing is known as to their behaviour in the body.

**72. Preparation of nucleoprotein.** Lymphatic glands of the ox or sheep, or the thymus of a calf are freed from fat, finely minced, ground with sand and extracted for twelve hours with ten times their weight of distilled water in a large bottle, a small amount of toluol or chloroform being added to prevent decomposition. The bottle should be shaken vigorously at frequent intervals to break up the gelatinous masses that sometimes form. The fluid is strained and centrifugalised to remove all debris (filtration being very slow). This fluid contains both nucleoprotein and nucleo-histone.

**73.** To a portion add dilute acetic acid till no more precipitate is produced, and place on the water-bath at 37° C. for a few minutes.

A heavy precipitate of nucleoprotein and nucleohistone is formed. Allow this to settle in a cylinder: pour or pipette off as much of the supernatant fluid as possible, and filter the remainder. Note that the precipitate is soluble in dilute alkalies and is reprecipitated by acidification; that it dissolves to an opalescent solution in excess of acetic acid (difference from mucin); and that it gives all the usual colour reactions of proteins.

74. To another portion add one-tenth of its volume of 2 per cent. calcium chloride and warm to  $37^{\circ}\text{C}$ . A white precipitate of nucleohistone is formed. Pour off the supernatant fluid, and to this fluid add dilute acetic acid drop by drop; a white precipitate of nucleoprotein is produced.

75. Precipitate the nucleoprotein and nucleohistone from the remainder of the fluid by means of acetic acid as in Ex. 73. Collect the precipitate on a filter paper, allow it to drain well, and then transfer it by means of a spatula to a small thimble-shaped porcelain capsule. Heat carefully, first to drive off the water, and then to carbonise the residue. Add one-third of a crucible full of fusion mixture ( $\text{K}_2\text{CO}_3$  two parts,  $\text{KNO}_3$  one part), and heat as strongly as possible till the mass fuses. Allow the melt to cool, and extract it with nitric acid (diluted with an equal quantity of distilled water) till the mixture no longer effervesces. Filter: treat the filtrate with about one-tenth of its volume of strong nitric acid and one-third its volume of ammonium molybdate; boil for two minutes. A yellow precipitate of ammonium phospho-molybdate separates out, often on the sides of the vessel. The phosphorus of the nucleic acid has been oxidised to phosphoric acid.

76. **Preparation of thymus nucleic acid.** (After W. Jones.) To a boiling mixture of 2 litres of water, 100 grms. sodium acetate and 23 grms. of caustic soda, add in small successive portions 1 kilo. of trimmed and finely ground calves thymus. Immerse the vessel for two hours in boiling water, stirring occasionally. Dilute with one-third volume of water and make faintly but distinctly acid to litmus with 50 per cent. acetic acid. The amount of acid required is usually about 100 cc., but the final additions must be made extremely cautiously until a point is reached which allows of good filtration.

If a portion does not filter well after being boiled and dried on a paper heated with boiling water, the point must be reached by the addition of more acetic acid or of caustic soda. Now boil the bulk and filter, using a hot water funnel. Concentrate the filtrate on a water bath to about 750 cc., and pour the warm solution slowly into 1 litre of 95 per cent. alcohol in a large beaker. Allow the mixture to stand overnight. The precipitated sodium nucleate settles to a spongy white mass. Pour off the supernatant fluid and squeeze out the remainder as far as possible by means of a spatula. Wash by decantation first with 80 per cent., and then with 95 per cent. alcohol. Squeeze out the last wash fluid as much as possible and transfer to a flask with 300 cc. of hot water, and heat on the water bath for 30 minutes. Add 10 cc. of 20 per cent. caustic soda, and filter from insoluble phosphates, using a hot water funnel. Acidify with acetic acid and pour into 700 cc. of 95 per cent. alcohol. Allow to stand over-night, wash by decantation with alcohol of increasing strength, and grind in a mortar with absolute alcohol until it has crumbled into a fine white powder. Transfer to a filter with absolute alcohol and dry in a sulphuric acid desiccator. The product should weigh over 30 grms., and consists of the soluble sodium salt of thymus nucleic acid.

A 4 to 5 per cent. solution in warm water becomes gelatinous at room temperature, the viscosity being decreased both by acetic acid and sodium hydroxide.

#### 77. Preparation of Guanine and Adenine from Nucleic Acid.

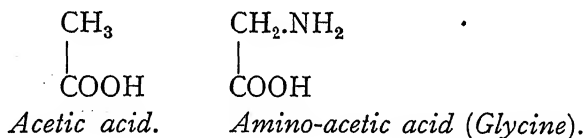
Heat on a boiling water bath 50 grams. of commercial yeast nucleic acid for two hours with 200 cc. of 10 per cent. sulphuric acid in a flask fitted with a reflux condenser. Treat the hot fluid with strong ammonia. Guanine is precipitated. Continue to add the ammonia till the neutral point is reached, and then add an excess of 2 per cent. of the reagent. Allow to cool and filter. Reserve the filtrate A. Wash the guanine with 1 per cent. ammonia, adding the washings to A. Suspend the guanine in boiling water and dissolve in a minimal amount of 20 per cent. sulphuric acid. Add a small amount of good charcoal, boil, and filter. Add ammonia as before to precipitate the guanine. Filter, dry at 40° C., and dissolve in 20 to 25 times its weight of boiling 5 per cent. hydrochloric acid. Upon

cooling the solution deposits needle clusters of guanine chloride,  $C_5H_5N_5O.HCl.2H_2O$ . The filtrate A is filtered again if necessary, and made faintly acid with 20 per cent. sulphuric acid. Boil and add 10 per cent. copper sulphate. The adenine cuprous compound is precipitated. Filter and wash. Suspend in water and decompose by sulphuretted hydrogen. Filter from copper sulphide and evaporate to dryness on the water bath. Dissolve in the smallest possible amount of hot 5 per cent. sulphuric acid, and allow to cool. Adenine sulphate  $(C_5H_5N_5)_2H_2SO_4.2H_2O$  is obtained in crystalline form.

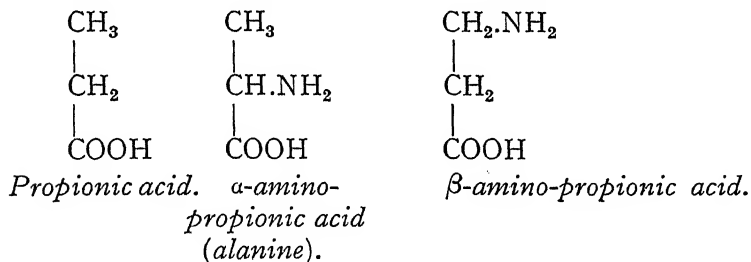
## CHAPTER IV.

### THE PREPARATION AND PROPERTIES OF CERTAIN AMINO-ACIDS.

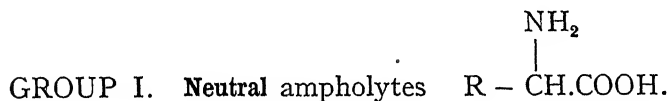
Amino-acids are compounds in which a H atom of an alkyl group of an organic acid has been replaced by an amino-group.



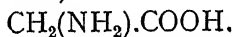
All the physiological amino-acids have the amino group attached to the same carbon atom as that to which the carboxylic group is attached, *i.e.* they are  $\alpha$ -amino-acids.



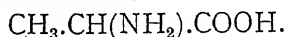
The following are the most important amino-acids that have been obtained from proteins by hydrolysis.



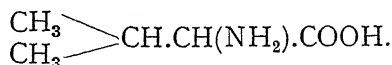
Glycine (amino-acetic acid)



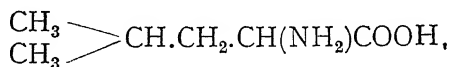
Alanine ( $\alpha$ -amino-propionic acid)



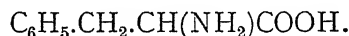
Valine ( $\alpha$ -amino-iso-valeric acid)



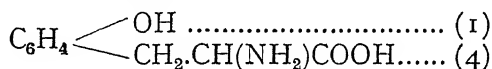
Leucine (isobutyl-amino-acetic acid)



Phenyl-alanine



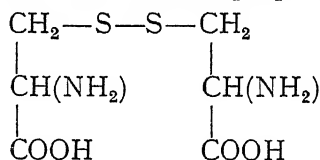
Tyrosine ( $p$ -oxy-phenyl-alanine)



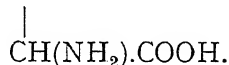
Tryptophane ( $\beta$ -indol alanine)



Cystine (dicysteine or di- $\beta$ -thio- $\alpha$ -amino propionic acid)

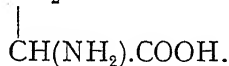


GROUP II. Acid ampholytes.



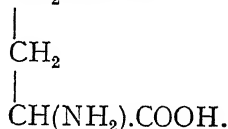
Aspartic acid

(amino-succinic acid)



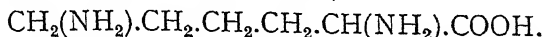
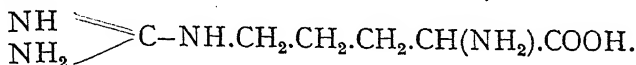
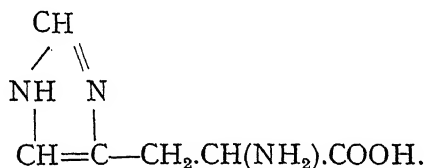
Glutaminic acid

(amino-glutaric acid)





## GROUP III. Basic ampholytes.

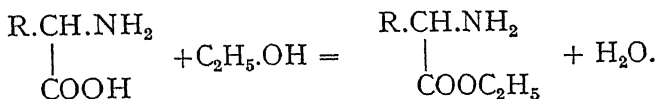
Lysine ( $\alpha, \epsilon$ , di-amino caproic acid)Arginine ( $\delta$ -guanidine- $\alpha$ -amino valeric acid)Histidine ( $\beta$ -iminazole-alanine)

## General reactions of the amino-acids.

1. They form two classes of salts :

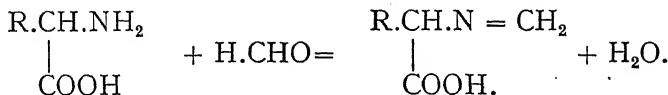
- (a) With acids, owing to the presence of the  $-\text{NH}_2$  group (see Ex. 80).  
 (b) With bases, owing to the presence of the  $-\text{COOH}$  group (see Ex. 79).

2. When dissolved in alcohol and saturated with dry hydrochloric acid they form esters, which are bases (see Ex. 78).



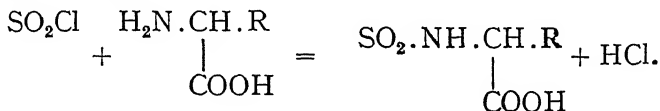
This reaction is of considerable importance, as Fischer's method of separation of the amino-acids is based on the fractional distillation of the esters.

3. They combine with aldehydes to form methylene compounds, which are acids (see Ex. 260).



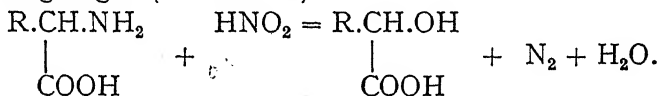
This reaction is the basis of Sørensen's method of estimating the production of amino-acids during tryptic digestion.

4. They form insoluble crystalline compounds with  $\beta$ -naphthalene-sulphochloride.



5. They form moderately soluble copper salts. With the exception of tryptophane (see p. 95) these can be readily crystallised from boiling water. The tryptophane copper salt is characteristically insoluble, even in boiling water. In the presence of even traces of other amino-acids it becomes soluble in the solution of their copper salts.

6. They are acted upon by nitrous acid, yielding nitrogen gas (see Ex. 81).



This important reaction is the basis of Van Slyke's gasometric method for the estimation of amino-acids in blood and tissues.

7. They are optically active with the exception of glycine, which does not contain an asymmetric carbon atom (see p. 147).

**Methods of separation.** The proteins can be hydrolysed by boiling acids, boiling alkalis, or by the action of certain enzymes, *e.g.* trypsin. The products are then separated by:—

I. Fractional *crystallisation* of the amino-acids, or of their hydrochlorides. In this way tyrosine, leucine, cystine, and glutaminic acid hydrochloride are obtained.

II. Fractional *precipitation*, *i.e.* by adding a reagent to the mixture which forms an insoluble compound with only one or a few of the substances present. In this way, by the use of mercuric sulphate, tryptophane was first isolated, and cystine and tyrosine can also be obtained; by the use of mercuric chloride histidine is separated.

III. Fractional *distillation* of the esters. This method, introduced by Emil Fischer, led to the discovery of several of the amino-acids, and serves for the quantitative estimation of some of them.

Percentage amounts of some amino-acids in certain proteins :

|                  | ALBUMIN<br>(Serum) | GLOBULIN<br>(Serum) | CASEIN<br>(Cow) | GLIADIN<br>(Wheat) | GELATIN     | ELASTIN | KERATIN<br>(Horse hair) | GLOBIN<br>(Hæmoglobin) |
|------------------|--------------------|---------------------|-----------------|--------------------|-------------|---------|-------------------------|------------------------|
| Glycine ..       | 0                  | 3.5                 | 0               | 0.4                | <b>16.5</b> | 25.8    | 4.7                     | —                      |
| Alanine ..       | 2.7                | 2.2                 | 0.9             | 2.3                | 0.8         | 6.6     | 1.5                     | 4.2                    |
| Leucine ..       | 20.0               | 18.7                | 10.5            | 6.0                | 2.1         | 21.4    | 8.0                     | <b>29.0</b>            |
| Tyrosine ..      | 2.1                | 2.5                 | <b>4.5</b>      | 1.8                | 0           | 3.9     | 3.2                     | 1.3                    |
| Tryptophane ..   | +                  | +                   | <b>1.5</b>      | 1.0                | 0           | —       | +                       | +                      |
| Cystine ..       | 0.3                | 0.7                 | 0.0             | —                  | 0           | —       | <b>11.0</b>             | 0.3                    |
| Aspartic acid .. | 1.5                | 2.5                 | 1.8             | 0.9                | 0.6         | +       | 0.3                     | 4.4                    |
| Glutaminic acid  | 8.0                | 8.5                 | 21.8            | <b>34.5</b>        | 0.9         | 0.3     | 3.7                     | 1.7                    |
| Lysine ..        | —                  | —                   | 5.8             | 0                  | 3.9         | —       | —                       | 4.3                    |
| Arginine ..      | —                  | —                   | 4.8             | 3.0                | 8.5         | 0.3     | —                       | 5.4                    |
| Histidine ..     | —                  | —                   | 2.6             | 1.2                | 0.4         | —       | —                       | <b>11.0</b>            |

The figures in heavy type draw attention to the reason why certain proteins are used for the preparation of particular amino-acids.

## 78. Glycine.

A *The preparation of glycine ester hydrochloride from gelatin.*

- (i.) To 300 cc. of pure concentrated hydrochloric acid in a round-bottomed flask add 100 grams. of ordinary glue.
- (ii.) Heat on a boiling water bath, with occasional shaking, until the glue has dissolved.

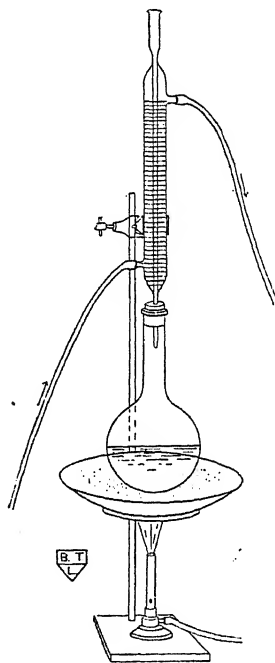


Fig. 7. Heating on a sand bath under a reflux condenser.

- (iii.) Boil the mixture on a sand bath under a reflux condenser for four hours (see fig. 7).
- (iv.) Transfer the dark product to a litre distilling flask, and distil off the acid as completely as possible *in vacuo*, using the apparatus shewn in fig. 8. The fluid is placed in flask A, which is immersed in a water bath maintained

at  $45^{\circ}$  to  $50^{\circ}$  C. Join this flask to another distilling flask *B*, by a tight-fitting rubber stopper. Connect the side neck, *D*, of this to an exhaust pump, and put the pump in action. The screw clamp, *C*, is on a piece of

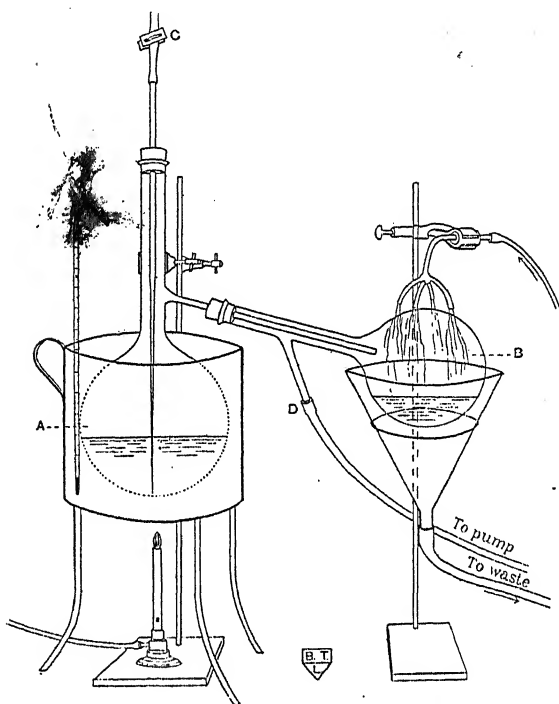


Fig. 8. Distillation *in vacuo*.\*

pressure tubing attached to a glass tube drawn to a fine capillary that passes to the bottom of the flask. The screw must be tightened until the bubbles of air pass through the fluid at such a rate that they cannot quite be counted. The flask *B* is cooled by means of a stream of cold water, which passes on to it by means of a double T-piece. In the absence of this it is advisable to place a piece of filter paper on the flask to keep the stream of water well spread.

\* For alternative method see p. 99.

- (v.) When as much acid as possible has been removed, slowly open the screw *C* to abolish the vacuum. Cautiously disconnect the rubber tubing at *D* before turning off the pump. With the pump connexions shewn in fig. 9, the abolition of the partial vacuum is readily accomplished without danger.

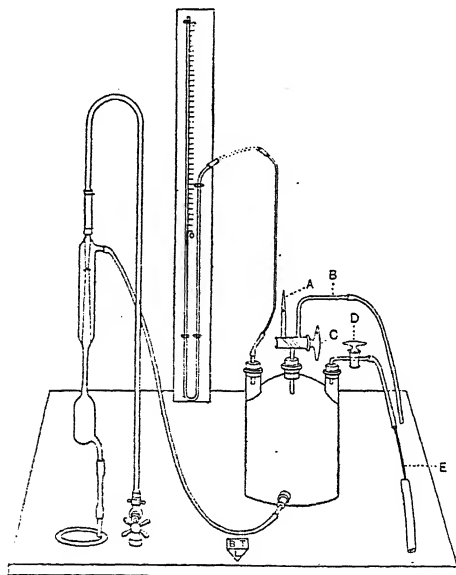


Fig. 9. Connexions of vacuum pump in author's laboratory. With the tap *C* in the position shewn a small amount of air is admitted through the narrow tube *A*. When *C* is turned through a right angle, full suction through *E* is obtained.

- (vi.) Disconnect the apparatus and stopper the side neck of *A* with a cork.
- (vii.) To the thick viscous mass in the flask add 500 cc. of absolute alcohol and heat on a water bath under a reflux condenser until it has dissolved.
- (viii.) Allow the solution to cool somewhat, add 3 to 5 grms. of a good quality animal charcoal, boil on the water bath for 10 minutes, and filter hot into a flat-bottomed litre flask.

- (ix.) Cool the filtrate, first under the tap, and then with ice, and pass in a stream of dry hydrochloric acid gas (see fig. 10) until the fluid is saturated.

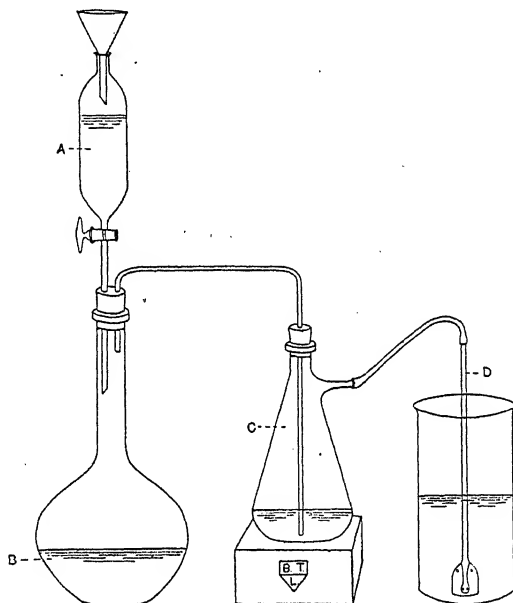
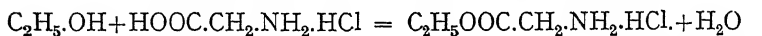


Fig. 10. Apparatus for saturating a fluid with dry hydrochloric acid gas.

B is concentrated hydrochloric acid. A is concentrated sulphuric acid, which is allowed to drop slowly into the flask. C contains concentrated sulphuric acid to dry the gas. The dry gas can be introduced into the fluid by means of a Folin absorption tube, D, though this is not usually necessary.

- (x.) Boil on a water bath under a reflux condenser for 30 minutes, cool thoroughly, and allow it to stand over-night in a refrigerator. The glycine ester hydrochloride generally separates as a mass of colourless needles. Should this not occur, it is advisable to "sow" the fluid with a small quantity of the crystals obtained from another preparation, or rub the sides of the vessel with a glass rod. [A second crop of crystals can often be obtained by concentrating in vacuo and repeating processes (ix.) and (x.).]

- (xi.) Filter on a small Buchner funnel (fig. 11), wash with a little ice cold absolute alcohol, and dry in a desiccator.



Glycine hydrochloride.      Glycine ester hydrochloride

*Yield:* 10 to 15 grams.

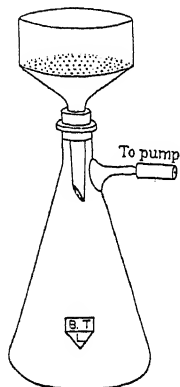


Fig. 11. Buchner funnel and filtering flask.

B. *The conversion of glycine ester hydrochloride into glycine.*

- (i.) Weigh the glycine ester hydrochloride, place it in a 250 cc. round-bottomed flask and add 10 cc. of water.
- (ii.) Add 100 cc. of ether and cool in a freezing mixture.
- (iii.) Gradually add 33 per cent. caustic soda, shaking well during the addition, until the aqueous layer is neutral to litmus. About 0.8 cc. are required for every gram of the hydrochloride.
- (iv.) Add powdered potassium carbonate, shaking vigorously between whiles, until the watery layer is a paste.

By treatment with alkali the glycine ester hydrochloride is converted into glycine ester, which is soluble in ether.

- (v.) Pour off the ether, filter it, and place it in a stoppered flask.
- (vi.) Add another 50 cc. of ether to the residue, shake well, remove and filter the ether, adding it to that in the stoppered flask. Repeat this once more.
- (vii.) Dry the ether by shaking for 5 to 10 minutes with about 20 grams of anhydrous potassium carbonate. Decant the ethereal solution and remove the last traces of water by means of anhydrous sodium sulphate. This is prepared by strongly heating 25 grams. of sodium sulphate in a porcelain dish and cooling the warm melt in a desiccator. Allow the cool, finely powdered, sulphate to stand with the ether for at least six hours.

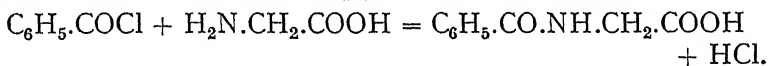


- (viii.) Filter off the ether and transfer it to a distilling flask. Connect this to another distilling flask as shewn in fig. 8.
- (ix.) Distil off the ether under reduced pressure, the receiving flask being thoroughly cooled (best done by packing with ice).
- (x.) When all the ether has been removed, change the receiving flask and distil over the glycine ester, at a temperature of  $44^{\circ}$  C. and a pressure of 11 mm. mercury. The receiving flask must be well cooled.
- (xi.) Transfer the distillate to a round-bottomed flask, add 10 times its volume of water, and boil on a sand bath under a reflex condenser until the alkaline reaction has disappeared.
- (xii.) Concentrate the solution in an evaporating basin on the water bath. Crystals of glycine are obtained.

**Properties of Glycine.** It crystallises from water in hard, flattened, colourless prisms. On heating, it becomes brown at  $228^{\circ}$ , and melts at  $232^{\circ}$ – $236^{\circ}$ . The crystals have a sweet taste, from which fact the original name of glyocoll was derived ( $\gamma\lambda\upsilon\kappa\acute{\upsilon}\varsigma$ , sweet :  $\kappa\acute{o}\lambda\lambda\alpha$ , glue). It is readily soluble in water (1 part of glycine in 4.3 parts of cold water). It is insoluble in absolute alcohol, and in ether. When boiled with *concentrated* alkali ammonia is evolved. On treating the residue with hydrochloric acid, hydrocyanic acid is evolved, and oxalic acid is found to be present.

Aqueous solutions give a red colour with ferric chloride, similar to that given by acetic acid.

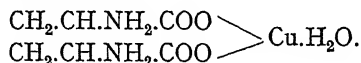
On shaking an aqueous solution with benzoyl chloride and sodium carbonate, hippuric acid is formed.



*Benzoyl chloride.*      *Glycine.*                      *Hippuric acid.*

**79. Preparation of the copper salt of glycine.** To a solution of about 0.5 gram. of glycine in about 40 cc. of distilled water, add an excess of freshly precipitated, well washed cupric hydroxide.

Boil for 5 minutes and filter. Concentrate the filtrate in an evaporating basin on a boiling water bath, and set the dish aside. Fine blue needles of the copper salt are formed, having the composition



NOTE.—The copper hydroxide is prepared by adding 10 cc. of 20 per cent. copper sulphate to about 100 cc. of distilled water, and stirring in 16 cc. of N. sodium hydroxide, previously diluted with about 300 cc. of water. The precipitate is filtered and very thoroughly washed with cold distilled water until neutral to litmus.

### 80. Glutaminic Acid.

#### A. Preparation of Glutaminic acid hydrochloride.

- (i.) To 100 grams. of gluten flour\* in a 500 cc. round bottomed flask add 300 cc. of pure concentrated hydrochloric acid, and heat on the water bath until the gluten has dissolved.
- (ii.) Add 20 grams. of good decolourising charcoal, to remove the dark "humins substance" that is formed during the subsequent hydrolysis.
- (iii.) Boil on a sand bath under a reflux condenser for 6 hours.
- (iv.) Dilute with an equal volume of water and filter.
- (v.) Evaporate the filtrate *in vacuo* to about 150 cc. (see pages 73 and 94).
- (vi.) Transfer the residue to a 300 cc. Erlenmeyer flask, cool thoroughly, and saturate with dry hydrochloric acid gas (see fig. 10).
- (vii.) Allow the flask to stand in the ice chest. After 24 to 48 hours a mass of crystals of glutaminic acid hydrochloride separates. Add an equal volume of ice cold alcohol.
- (viii.) Filter on a Buchner funnel through a piece of well washed linen (handkerchief) cut to fit the funnel. Drain the mother liquor away as completely as possible.
- (ix.) Wash the crystals with small amounts of ice cold concentrated hydrochloric acid.

*Yield:* about 40 grams.

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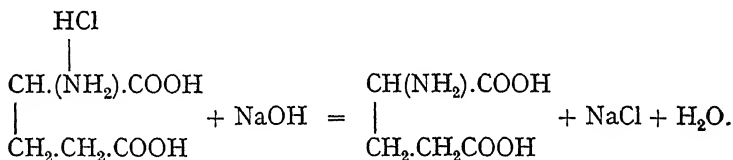
\* This can be obtained from Messrs. Bishop and Brooke, 21, Cock Lane, Snow Hill, London, E.C.

B. *Recrystallisation of the hydrochloride.*

- (i.) Dissolve the crystals in about 100 cc. of water, boil with a sufficiency of decolourising charcoal, and filter.
- (ii.) Saturate the cooled filtrate with dry hydrochloric acid gas, and allow to stand in the ice chest over-night.
- (iii.) Add an equal volume of ice cold absolute alcohol and filter through linen on a Buchner funnel.
- (iv.) Dry in a vacuum desiccator over potash and sulphuric acid.

C. *Preparation of glutaminic acid from the hydrochloride.*

- (i.) Weigh the pure, dry, hydrochloride and dissolve it in a minimal amount of water. Add 5.44 cc. of N.NaOH for every gram, this being the amount required to remove the HCl according to the following equation.

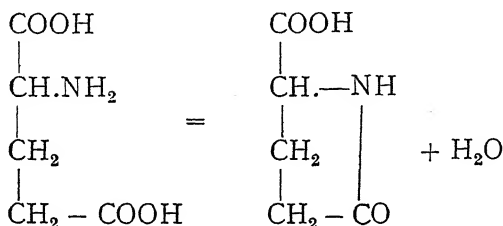


- (ii.) Evaporate the solution *in vacuo* at 40° to 50° to reduce the volume to 60 to 100 cc.
- (iii.) Transfer the warm solution to a beaker, and allow it to stand over-night in the ice chest.
- (iv.) Filter off the crystals on a Buchner, wash with a little cold water, and dry.

*Yield:* 18 to 20 grams.

**Properties of Glutaminic Acid.** It crystallises from water in rhombic tetrahedra, which on rapid heating melt at 213°. It dissolves in about 100 parts of cold water, but is much less soluble in alcohol. Since it contains two carboxyl and only one amino-group, its aqueous solutions are markedly acid to litmus. The hydrochloride forms

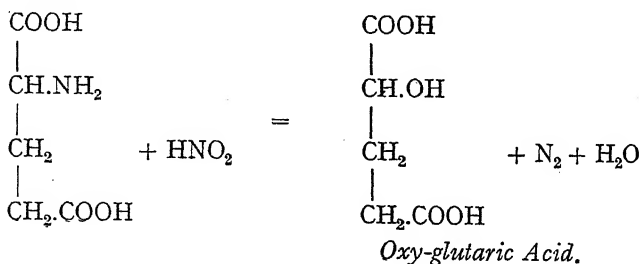
triclinic tables, which melt at about  $193^{\circ}$ . This salt is readily soluble in water, but only very slightly soluble in concentrated hydrochloric acid. The calcium salt is quantitatively precipitated by strong alcohol, provided that the solution be sufficiently concentrated (25 to 30 per cent.). On boiling an aqueous solution of the hydrochloride, it is largely converted into the internal anhydride, pyrrolidone carboxylic acid.



This change does not take place in the presence of strong hydrochloric acid, nor during concentration *in vacuo* at  $40^{\circ}$  to  $45^{\circ}$ . Glutaminic acid is especially abundant in vegetable proteins. It is for this reason that gluten flour, consisting of gliadins and glutelins, is used for its preparation.

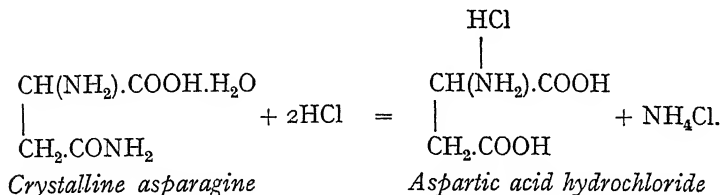
Glutaminic acid is dextrorotatory,  $[\alpha]_D$  in water  $= +12^{\circ}$ . In 10 per cent. hydrochloric acid  $[\alpha]_D = +31^{\circ}$ .

**81. Action of nitrous acid.** To a solution of glutaminic acid in water add a solution of nitrous acid, obtained by acidifying a strong solution of potassium nitrite with acetic acid. An evolution of nitrogen gas occurs.



**Aspartic Acid.**

82. **Preparation from Asparagine.** Asparagine is the amide of aspartic acid, and it is converted to the acid by hydrolysis with acids.



Mol.Wt. = 150.

The hydrochloride is converted to aspartic acid by the addition of the calculated amount of sodium hydroxide.

- (i.) Into a 500 cc. round-bottomed flask introduce 15 grams. of crystalline asparagine (1/10 gm. mol.) and 200 cc. of N.HCl (2/10 gm. mol.).
- (ii.) Boil gently on a sand bath under an efficient reflux condenser for 6 hours.
- (iii.) Cool and add 100 cc. of N.NaOH (1/10 gm. mol.), shaking during the addition. Set aside to crystallise in a cool place. (This soda is to convert aspartic acid hydrochloride into free aspartic acid.)
- (iv.) Stir well and filter on a Buchner, and wash with small amounts of cold water. Reserve the filtrate and washings for obtaining copper aspartate, or a further crop of crystals by evaporation.
- (v.) Recrystallise by dissolving in the smallest possible amount of 50 per cent alcohol, filtering through a hot water funnel, and allowing to stand till quite cold.
- (vi.) Filter on a Buchner and dry in the air. Add the filtrate to that obtained in (iv.).

*Yield:* about 12 grams. of the recrystallised product.

**Properties of Aspartic Acid.** It crystallises in small rectangular plates. It dissolves in about 360 parts of cold water and 19 parts of boiling water. Like glutamic acid, its aqueous solutions are markedly acid to litmus. Solutions in alkalies are laevorotatory, those in hydrochloric acid are dextrorotatory.

The copper salt is very characteristic. It can be obtained by the method given in Ex. 79, or more readily by boiling a solution with some solid cupric acetate, and filtering the hot liquid. On standing, beautiful blue needles separate. This copper salt, which contains  $4\frac{1}{2}$  molecules of water of crystallisation, is so sparingly soluble that it serves for the estimation of aspartic acid.

### Cystine.

#### 83. Preparation from hair (or wool) by Folin's method.

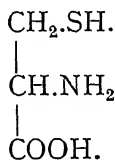
- (i.) Heat 500 cc. of pure concentrated hydrochloric acid in a litre round-bottomed flask on a water bath.
- (ii.) Add 250 grms. of human hair (the sweepings from a hair-dresser's shop), or of washed wool (a piece of a pure woollen blanket). If hair is used it must be added in portions of about 50 grams. at a time, the hot mixture being well agitated after each addition.
- (iii.) Boil under a reflux condenser on a sand bath for 5 to 6 hours, or until the mixture no longer yields the biuret reaction.
- (iv.) To the hot mixture add solid sodium acetate to remove the free hydrochloric acid. The point is reached when a drop of the mixture added to about 2 cc. of water gives a reddish violet or brown, and not a deep blue with a few drops of a dilute (0.2 per cent.) solution of Congo red. Usually 500 to 600 grams. of the solid are required. Allow the mixture to stand over-night.
- (v.) Filter on a Buchner. The precipitate consists of cystine, together with a considerable amount of dirt and insoluble debris.

- (vi.) Transfer the precipitate to a porcelain beaker or dish, and boil with 150 cc. of 20 per cent. hydrochloric acid (by volume). Filter.
- (vii.) Boil the residue with another 100 cc. of the hydrochloric acid, filter, and mix the two filtrates.
- (viii.) Boil these with 5 grams. of good decolourising charcoal (see p. 390), and filter. If the filtrate is not practically colourless, the solution must be boiled with more charcoal until a colourless or light yellow fluid is obtained.
- (ix.) Add a hot, concentrated, filtered solution of sodium acetate until the free hydrochloric acid is removed.
- (x.) Allow to stand till quite cold.
- (xi.) Filter off the cystine, wash with cold water, then with alcohol, and dry in the air.

*Yield* : 8 to 10 grams.

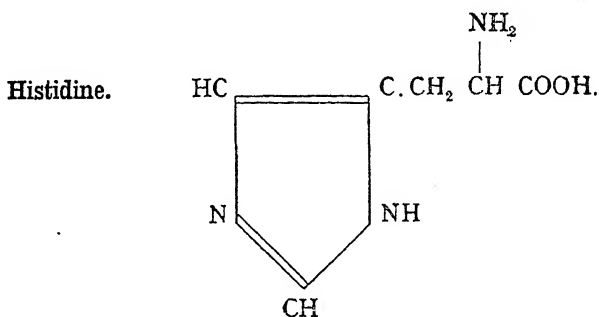
**Properties of Cystine.** It crystallises in characteristic hexagonal plates, which are only very slightly soluble in cold water (1 part in 8840 parts of water) and in alcohol. It dissolves readily in mineral acids, but is insoluble in acetic acid. In normal acid urine it is soluble to the extent of 1 part in 2000. It is readily soluble in dilute alkalies and in ammonia.

It is precipitated from its solution in sulphuric acid by mercuric sulphate (see p. 90). On reduction it yields cysteine



This is readily oxidised to cystine by atmospheric oxygen in ammoniacal solution.

84. Dissolve a small amount of cystine in one or two cc. of 5 per cent. sodium hydroxide. Add a drop or two of lead acetate and boil for a minute. The solution is darkened owing to the formation of lead sulphide (see Ex. 25).



**85. The preparation of histidine mono-hydrochloride.**

- (i.) Place 1 litre of defibrinated ox or sheep blood (or preferably 1 litre of the centrifuged corpuscular mass from blood) into a 2-litre, round-bottomed flask.
- (ii.) Add 500 cc. of pure concentrated hydrochloric acid, shaking well during the addition.
- (iii.) Heat on a boiling water bath, shaking at intervals, for 2 to 3 hours, until the precipitated blood proteins have re-dissolved.
- (iv.) Boil on a sand bath under a reflux condenser for 10 hours.
- (v.) Transfer to an evaporating basin and remove the greater part of the hydrochloric acid by evaporating on a boiling water bath in a flue chamber.
- (vi.) Add 40 per cent. caustic soda until the mixture is only slightly acid to litmus paper.
- (vii.) Allow to stand over-night, and filter on the pump. Wash the precipitate with hot water, adding the washings to the main filtrate.



- (viii.) Place the solution in an evaporating basin, make it distinctly alkaline by the addition of caustic soda, and boil for 30 to 60 minutes to remove ammonia. This is tested by holding a moist litmus paper in the vapour. The removal of ammonia is hastened by adding a small volume of alcohol.
- (ix.) Pour the solution into about 5 litres of water contained in a large vessel.
- (x.) Add a hot saturated solution of mercuric chloride in water until no further precipitate is obtained, keeping the solution sufficiently alkaline to ensure complete precipitation. Usually about 100 gms. of mercuric chloride are required.
- (xi.) Allow the mercury compound of histidine to settle over-night.
- (xii.) Syphon off the supernatant fluid.
- (xiii.) Filter off the precipitate on a large Buchner funnel and wash it with cold water.
- (xiv.) Transfer the precipitate to a porcelain dish and add hot hydrochloric acid (25 per cent. by volume) as long as any of the precipitate goes into solution, avoiding any large excess of acid.
- (xv.) Filter from the insoluble residue of calomel and wash this with cold water, adding the washings to the bulk of the fluid.
- (xvi.) Dilute the fluid to about 5 litres with distilled water.
- (xvii.) Dissolve 20 grams. of mercuric chloride in water and add this to the fluid.
- (xviii.) Make the fluid markedly alkaline by the addition of caustic soda.
- (xix.) Allow the voluminous precipitate to settle over-night.
- (xx.) Filter on the pump, drain, and wash thoroughly by grinding with water in a mortar and filter again.

- (xxi.) Grind the precipitate with a litre of water, transfer to a flask and decompose by means of sulphuretted hydrogen gas. As the precipitate is somewhat difficult to decompose it is necessary to pass the gas for 8 to 10 hours. It must not be assumed the decomposition is complete as soon as the precipitate has blackened. On no account must the solution be heated.
- (xxii.) Filter off the mercuric sulphide, wash it with small quantities of hot water, adding the washings to the bulk of the fluid.
- (xxiii.) Concentrate the solution to a syrup in an evaporating basin on a boiling water bath.
- (xxiv.) Whilst still hot, add boiling 97 per cent. alcohol, with continuous stirring, until there is a faint permanent turbidity. Allow to stand over-night.
- (xxv.) Filter off the crystals of histidine hydrochloride,  
 $C_6H_9N_3O_2.HCl, H_2O$ .
- (xxvi.) Repeat (xxiii.) to (xxv.) to obtain a second crop.

#### Recrystallisation.

- (i.) Dissolve the crystals in twelve times their weight of 65 per cent. alcohol, by heating on a boiling water bath under a reflux condenser.
- (ii.) Add a little charcoal and boil again.
- (iii.) Filter through a pleated paper on a hot water funnel, and allow the filtrate to cool. The histidine hydrochloride separates in the form of beautiful white glistening plates.

*Yield:* 6 to 8 grams.

**Properties of histidine.** Histidine is readily soluble in water, but very slightly soluble in alcohol. The aqueous solution has an alkaline reaction. It crystallises from alcohol in platelets, which melt with decomposition at about  $253^{\circ}C$ .

The most convenient salt for the isolation of histidine is the monohydrochloride. It is readily soluble in water.

It crystallises from aqueous alcohol in platelets, which melt at  $251^{\circ}$ — $252^{\circ}$  C.

Even in very dilute solution histidine gives a voluminous white precipitate with phosphotungstic acid. It is also precipitated by mercuric chloride in alkaline solution and by ammoniacal silver nitrate solution.

86. **Totani's reaction for histidine.** To a small knife point of the hydrochloride in a small beaker, add 2 cc. of 10 per cent. sodium carbonate. Dissolve a rather larger quantity of diazobenzene-sulphonic acid in 4 cc. of 10 per cent. sodium carbonate in a test-tube, and add this to the solution in the beaker. A dark red colour is produced (*Primary colouration*). Make the solution distinctly acid with strong hydrochloric acid. The solution becomes orange coloured. Add zinc dust and allow the reduction to proceed for about 15 minutes. Transfer a few cc. of the clear, colourless, supernatant solution to a test-tube, and render the solution alkaline by the addition of 25 per cent. ammonia. A characteristic golden yellow colouration is produced, which is permanent for a considerable time (*Secondary colouration*).

NOTE.—Tyrosine gives a primary colouration that is identical with that described above. The secondary colouration is, however, a bright rose red, which gradually changes to a reddish brown. The reaction should be tried with the two substances simultaneously.

### **Tryptophane.**

#### **87. Preparation.**

##### **A. Digestion of the Casein.**

- (i.) Weigh out 200 grams. of commercial casein.\*
- (ii.) Gradually stir this into 1 litre of cold distilled water in a large beaker, or enamelled vessel, avoiding the formation of lumps as far as possible.
- (iii.) Transfer the viscous mass to a Winchester quart bottle by means of a large funnel with a short wide neck.
- (iv.) Wash out the mixing vessel with a jet of hot water and transfer this to the bottle, washing the funnel down with some more hot water.

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\* "Insoluble Casein," i.e. casein without any added sodium carbonate or bicarbonate, is the best to use. It can be obtained from Messrs. Baird and Tatlock.

- (v.) Add more hot water to make the volume up to about 2 litres and shake vigorously.
- (vi.) Adjust the reaction to  $P_H$  = about 8.1. 10 cc. of the mixture is taken, treated with about 10 drops of cresol red and titrated with 0.2 N. NaOH from a microburette (or a 1 cc. pipette graduated in 1/100th cc.) until a reddish purple colour is obtained. The bulk is then treated with the corresponding amount of N. soda (*i.e.* 100 times the amount of 0.2 N. used). Should the volume required exceed 100 cc., 40 per cent. soda can be used, this being 10 N. The mixture is well shaken at frequent intervals after the addition of the soda. The reaction should now be alkaline to cresol red and acid to phenol phthalein.
- (vii.) Add 15 cc. of toluol (to prevent putrefaction) and 2 grams. sodium fluoride dissolved in about 10 cc. of hot water (to decrease the action of oxidases). Shake well.
- (viii.) Add 70 cc. of the pancreatic extract described on p. 212, or a corresponding amount of a commercial preparation of trypsin ("liquor pancreaticus") and mix well.
- (ix.) Clean the inside of the neck of the bottle with a cloth, insert a cork, shake again, and stand the bottle in a water bath or air thermostat at 37° to 40° C. for 4 days, shaking the bottle every day without removing the cork.
- (x.) After 4 days add another 50 cc. of the pancreatic extract, and allow the digestion to proceed for another 4 days, making 8 days in all.
- (xi.) Remove the bottle from the incubator, and allow it to stand at room temperature for 24 hours or longer.

B. *Filtration and acidification.*

- (i.) Filter off the precipitate, which consists of tyrosine, undigested casein, etc., reserving it for the separation of tyrosine described in Ex. 89.

- (ii.) Measure the filtrate and to every 86 cc. add 14 cc. of a 50 per cent. solution (by volume) of pure sulphuric acid. (This is prepared by gradually pouring 500 cc. of pure sulphuric acid into 500 cc. of distilled water, cooling thoroughly under the tap until the whole of the acid has been added. When quite cold the volume is made up to 1000 cc. with distilled water.) The mixture now contains 7 per cent. by volume of sulphuric acid.

C. *Separation of the mercuric sulphate precipitate.*

- (i.) Add 250 cc. of a 10 per cent. solution of mercuric sulphate in 7 per cent. sulphuric acid (by volume). Mix well, and allow to stand over-night. (The mercuric reagent is prepared by grinding 100 grams. of mercuric sulphate with 500 cc. of distilled water, to which 70 cc. of pure concentrated sulphuric acid has been added, adding distilled water to make a volume of 1000 cc., and filtering if necessary.)
- (ii.) Filter off the bulky yellow precipitate of the mercuric sulphate compound of tryptophane on a Buchner funnel, reserving the filtrate for Ex. 90.
- (iii.) Wash the precipitate on the Buchner with cold 5 per cent. sulphuric acid (by volume) to remove the tyrosine. It is not necessary to remove the tyrosine completely at this stage.
- (iv.) Wash the precipitate with distilled water to remove the greater part of the sulphuric acid.

D. *Decomposition of the mercuric sulphate precipitate.*

- (i.) Transfer the precipitate and paper to a wide-necked 500 cc. flask, washing the remainder of the precipitate in the Buchner into the flask by means of about 200 cc. of distilled water. Agitate thoroughly to get a good suspension of the precipitate.
- (ii.) Add 100 cc. of boiling water, to which 3 grams. of crystalline barium hydroxide has been added. Shake well.

- (iii.) Test the reaction of the solution by means of litmus paper. If it is still acid add a hot solution of baryta until alkaline.
- (iv.) Pass in a stream of sulphuretted hydrogen gas, shaking at intervals, until the mixture is fully saturated.
- (v.) Heat on the water bath to about  $50^{\circ}$  C., shake well, and pass in more of the  $\text{SH}_2$  if the odour of the gas is not perceptible.
- (vi.) Filter from the mixture of mercuric sulphide and barium sulphate.
- (vii.) Wash the precipitate with hot water, and squeeze the paper in a piece of muslin. Filter these washings, etc., through another small paper, and add them to the bulk of the fluid obtained in (vi.).

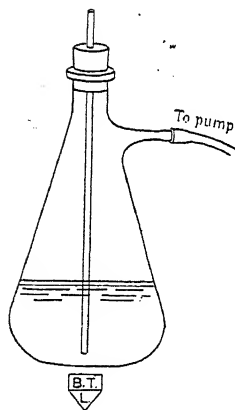


Fig. 12. Apparatus for removal of  $\text{SH}_2$  by means of an air current.

(viii.) Add a few drops of 5 per cent. sulphuric acid. If a white precipitate of barium sulphate is obtained, it indicates that an excess of baryta had been added, and that barium sulphide is present. Continue to add the dilute sulphuric acid until no further precipitate is obtained. Filter off the barium sulphate. If the addition of the dilute sulphuric acid does not cause a precipitate, proceed directly to:—

- (ix.) Remove the sulphuretted hydrogen by a strong current of air, using the apparatus shown in fig. 12. (It is preferable to remove the  $\text{SH}_2$  by distillation in vacuo at  $45^{\circ}$  C.)

*E. Removal of cystine and reprecipitation of the tryptophane.*

- (i.) Measure the fluid and add 14 cc. of 50 per cent. sulphuric acid for every 86 cc.

- (ii.) Cautiously add the mercuric sulphate reagent prepared as described in C (iii.) above. Add this until a slight definite precipitate is obtained. Usually about 15 cc. are required. Allow the mixture to stand for 10 minutes. Filter.
- (iii.) To the filtrate add 80 to 100 cc. of the mercuric reagent, and allow the mixture to stand over-night.
- (iv.) Filter on a Buchner funnel, wash with cold 5 per cent. sulphuric acid, and then thoroughly with water.
- (v.) Suspend the precipitate and paper in 50 cc. of water.
- (vi.) Add 2 grms. of barium hydroxide dissolved in 70 cc. of boiling water.
- (vii.) Decompose by  $\text{SH}_2$  and proceed as in D (iv.) to D (ix.), taking care to have only a very slight amount of free sulphuric acid present.

#### F. *Removal of sulphuric acid*

- (i.) Heat the fluid on a boiling water bath, and add a hot solution of baryta to a point when no further precipitation can be seen.
- (ii.) Filter a portion until a clear filtrate is obtained, and test with a drop or two of the baryta solution. If no precipitate is obtained, test another portion of the hot filtrate with a drop of dilute sulphuric acid. Should this fail to give a precipitate also, the correct point is reached, being that at which neither sulphuric acid nor baryta gives a precipitate. Baryta or sulphuric acid must be added until this condition is attained, the samples tested being added to the bulk. The process is much facilitated if the relative concentrations of the alkali and acid are roughly determined against one another. Filter to obtain a perfectly clear filtrate.
- (iii.) Add a single drop of 10 per cent. ammonia.

G. *Crystallisation.*

- (i.) Evaporate *in vacuo* (fig. 8) at a temperature of about 45° C. until the volume is reduced to rather less than 10 cc., best ascertained by measuring 10 cc. into the flask before the evaporation is commenced. (See page 99.)<sup>1</sup>
- (ii.) Disconnect the apparatus with the usual precautions. (See page 74.)
- (iii.) Heat for a short time on a boiling water bath, shaking the fluid round the flask to get as much as possible into solution.
- (iv.) Transfer to a small crystallising dish or beaker and add an equal volume of strong alcohol.
- (v.) Allow the dish to stand in a cool place over-night.
- (vi.) Filter off the precipitate, using a suction pump.
- (vii.) Wash out the flask and dish with small amounts of alcohol of increasing strengths, 65, 75, 85, and 95 per cent., using these for washing the crystals on the filter.
- (viii.) Dry the crystals in the air.

H. *Concentration of the mother liquors.*

- (i.) Evaporate the mixed mother liquors and alcoholic washings in a boiling water bath, adding strong alcohol from time to time, until the crystalline precipitate that forms at the edge does not redissolve in the body of the fluid when stirred.
- (ii.) Set the dish aside for at least an hour.
- (iii.) Filter off the crystals and wash with small amounts of alcohol of gradually increasing strength.
- (iv.) Dry in the air.

*Yield:* 0.6 to 2 grams.



### I. *Recrystallisation.*

- (i.) Transfer the crystals to a small flask, fitted with a reflux condenser.
- (ii.) Add a small amount of 70 per cent. alcohol, and heat in a boiling water bath. Very gradually add 70 per cent. alcohol (down the stem of the condenser) until the crystals have just dissolved. Add a large "knife-point" of decolourising charcoal and heat for five minutes.
- (iii.) Disconnect and rapidly filter through a small hot funnel into a small beaker. Allow to stand for at least an hour.
- (iv.) Filter on the pump, wash with 75, 85, and 95 per cent. alcohol. Dry in a vacuum desiccator, or in a warm oven at 80° to 90° C. There is a considerable loss on recrystallisation.

**Properties of Tryptophane.** It crystallises from aqueous alcohol in white glistening, six-sided plates. It is moderately soluble in cold water, but freely soluble in hot water. It is only sparingly soluble in absolute alcohol. On heating it changes colour at 220°, browns at 240°, and melts at 252° C. On heating still further, there is first an evolution of carbon dioxide, and then the formation of indol and skatol.

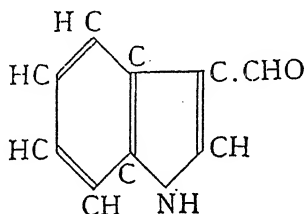
Tryptophane is optically active, being laevorotatory in aqueous, but dextrorotatory in acid or alkaline solution.

It gives colour reactions with a great variety of substances. The most important of these is the reaction with glyoxylic and sulphuric acids (see Ex. 23). The investigation of the cause of the similar colour reaction given by proteins led to the isolation of the amino-acid. It also gives colour reactions with most aldehydes in the presence of strong hydrochloric or sulphuric acids containing a trace of an oxidising substance, like ferric chloride. All these reactions are given by tryptophane when it is combined in a protein molecule.

Free tryptophane gives a red-rose colour when treated with bromine water, the colouring matter being soluble in

amyl or butyl alcohol. This reaction is not given by combined tryptophane.

Tryptophane is somewhat unstable. It is rapidly destroyed by boiling acids, especially in the presence of carbohydrates, yielding a dark, so-called "humin substance," or "melanin." It is oxidised by certain metallic salts, like copper sulphate, silver nitrate, gold chloride and ferric chloride, yielding indol aldehyde



and other substances. On heating on an open water bath in aqueous solution it suffers decomposition. For this reason it is necessary to concentrate *in vacuo*, or to add considerable quantities of alcohol during the evaporation. It is much more stable in alkaline solutions and, in the presence of other amino-acids, can be boiled with strong baryta for days without appreciable loss. It must be noted, however, that the substance obtained after baryta hydrolysis is racemic (see p. 152).

In dilute sulphuric acid tryptophane gives a lemon-yellow precipitate with mercuric sulphate, the precipitate being a compound of tryptophane sulphate with mercuric sulphate. The precipitating effect of phosphotungstic acid on tryptophane seems to vary with the quality of the acid; some samples only give a precipitate in concentrated solutions, others none at all, whilst some preparations precipitate it completely even from relatively dilute solutions, especially in the presence of other amino-acids. It is not precipitated by lead acetate nor by mercuric chloride in neutral solution.

88. A. To a small knife point of tryptophane dissolved in about 3 cc. of water, cautiously add bromine water. A rose-red or

reddish-violet colour is produced, which turns yellow on adding an excess of bromine. If the addition of the bromine water be stopped when the red colour has reached its maximum intensity, it will be found that the coloured product can be shaken out with a few cc. of amyl or butyl alcohol.

B. To a small knife point of tryptophane add a few cc. of "reduced oxalic acid" (see Ex. 23). Add an equal volume of pure sulphuric acid and mix. A beautiful purple colour is produced.

C. To a small knife point of tryptophane add about 1 cc. of water, 2 drops of a 5 per cent. solution of cane sugar, and 5 cc. of pure hydrochloric acid. Boil for about a minute. A deep purple solution is obtained.

D. Dissolve about 0.05 gram. of tryptophane in 5 to 10 cc. of water by boiling in a test tube. Add as much freshly prepared, washed copper hydroxide (see note to Ex. 79) as will go on a large spatula and boil for 1 minute. Filter hot. The filtrate is not blue and gives no reactions for tryptophane. The precipitate contains the copper salt of tryptophane, which is characteristically insoluble except in the presence of even traces of other amino-acids. It is then soluble in their copper salts.

### Tyrosine.

Tyrosine is the least soluble of the amino-acids, and for that reason is easily obtained from proteins. When casein is digested by trypsin, under the conditions described in Ex. 87, it generally happens that a considerable amount separates as a chalky white precipitate in 6 to 10 days. The amount separating varies with the activity of the ferment preparation, and on the particular sample of casein employed. If a good yield of tyrosine is especially desired it is advisable to concentrate the filtrate obtained in Ex. 87, B (i.) to about one-fourth, allow to cool over-night, filter off the precipitate on the pump, and purify by the method described below. The concentrated filtrate can be diluted, and used for the isolation of tryptophane, but owing to its unstability, the yield of this latter amino-acid is very apt to be poor.

**89. Preparation of Tyrosine from Casein.**

- (i.) Use the mixed mass of calcium phosphate, undigested casein, tyrosine, etc., obtained in Ex. 87, B (i.).
- (ii.) Boil the precipitate with about 250 cc. of water to which has been added 5 cc. of pure sulphuric acid. The tyrosine dissolves in the acid, whilst a considerable amount of the protein residue remains insoluble.
- (iii.) Filter through a pleated paper, passing the filtrate back through the paper until it is clear. Filtration is apt to be rather slow.
- (iv.) Heat the filtrate on a boiling water bath, and add 10 cc. of strong ammonia. The reaction should now be acid to litmus paper. Cautiously neutralise by the addition of ammonia and allow to cool. Tyrosine crystallises out, generally contaminated with calcium phosphate, etc.
- (v.) Filter off the tyrosine on the pump. Suspend it in about 300 cc. of water in a flask, boil, and add 5 cc. of strong ammonia and boil for 15 minutes.
- (vi.) Filter from the calcium phosphate.
- (vii.) Neutralise the fluid with 5 per cent. sulphuric acid and allow to stand.
- (viii.) Filter off the tyrosine on the pump, and wash well with cold water. Wash with a little alcohol, and dry in the steam oven or in a warm incubator.

**90. The separation of tyrosine by fractional precipitation.**

- (i.) Treat the filtrate obtained in Ex. 87, C (ii.) with 5 volumes of tap water in a large vessel, mix well, and allow to stand over-night. Owing to the reduction in the concentration of sulphuric acid the tyrosine is precipitated as a compound with mercuric sulphate.
- (ii.) Syphon off the supernatant fluid and filter the precipitate on a Buchner. Wash well with water.
- (iii.) Suspend the precipitate in about 200 cc. hot water and decompose by a stream of sulphuretted hydrogen gas.
- (iv.) Boil and filter. Tyrosine is left in solution in diluted sulphuric acid.

- (v.) Neutralise to litmus paper with ammonia, and allow to stand. Filter off the crystals of tyrosine, wash with cold water, and dry in a warm oven.

**Properties of tyrosine.** It crystallises from water in white needles which are characteristically arranged in sheaves. It is only very slightly soluble in cold water (1 part in 2490 parts of water at 17° C.), but more soluble in hot water (1 part in 150 parts). It is insoluble in ether, acetone and absolute alcohol. It is readily soluble in dilute alkalis and dilute mineral acids, but it is only very slightly soluble in dilute acetic acid, and practically insoluble in glacial acetic acid. Its melting point is rather indefinite, but on rapid heating is 314° to 318°. It is laevorotatory in aqueous acid and alkaline solutions.

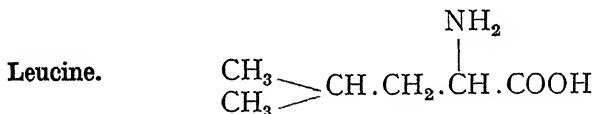
On being heated in a tube it loses CO<sub>2</sub>, and is converted to *p*-oxyphenylethylamine. It is not precipitated by phosphotungstic acid. It is precipitated by mercuric sulphate, the precipitate being soluble in dilute sulphuric acid.

91. **Mörner's reaction.** To a few cc. of Mörner's reagent add a trace of tyrosine and boil. A green colouration is produced.

NOTE.—The reagent is prepared by mixing 1 cc. of formalin with 45 cc. of water and cautiously adding 55 cc. of strong sulphuric acid.

92. **Millon's reaction.** To a trace of tyrosine add a few cc. of water and a drop of dilute sulphuric acid and boil. Cool the solution and add a few drops of Millon's reagent. A precipitate is not obtained. Heat. A red colouration is obtained.

NOTE.—For the preparation of Millon's reagent, see Ex. 22. In the presence of 5 per cent. sulphuric acid the red colour is produced in the cold.



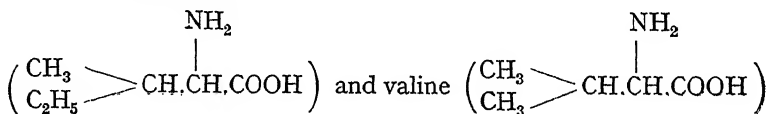
*α*-amino-caproic acid (*α*-amino-iso-butyl-acetic acid).

Leucine can be obtained by fractional crystallisation from a protein digest, being separated by concentration of the mother liquors left after the isolation of tyrosine. The

following exercise is suggested owing to the great preponderance of leucine over tyrosine in the proteins employed.

93. *Preparation of leucine from blood.*

- (i.) To 1 litre of defibrinated blood in a 2 litre flask, gradually add 150 cc. of pure sulphuric acid, shaking well during the addition. A semi-solid mass of coagulated protein is obtained.
- (ii.) Heat on a boiling water bath for 12 to 16 hours, shaking well at intervals.
- (iii.) Add some pieces of a broken porous pot, and heat to boiling on a large sand bath. It is necessary to start with the fluid hot from a water bath and to repeatedly shake the mixture until it boils. Otherwise there is a risk of the flask breaking. The mixture must be boiled for 10 to 14 hours.
- (iv.) To the hot fluid add a hot solution of baryta until the mixture is alkaline to litmus. About 500 grams. of baryta in about  $1\frac{1}{2}$  litres of boiling water are usually necessary.
- (v.) Filter on a Buchner funnel.
- (vi.) Make the filtrate acid to litmus with dilute sulphuric acid. Concentrate in a porcelain basin over a free flame to about 500 cc. and filter.
- (vii.) Render the filtrate faintly alkaline to litmus by the addition of ammonia and concentrate on a boiling water bath until a crystalline crust has formed. Allow to cool over-night.
- (viii.) Filter on a small Buchner funnel, pressing the mass of crystals firmly with a pestle to remove as much of the mother liquors as possible. The filtrate may be further concentrated and a second crop of crystals obtained.
- (ix.) Recrystallise from 70 per cent. alcohol, as described in Ex. 87, I. The product is apt to be contaminated with isoleucine



**Properties of leucine.** Pure leucine is only very slightly soluble in cold water, but when it is contaminated with other amino-acids it is easily soluble. It is insoluble in absolute alcohol, but is soluble in hot dilute alcohol, and can be freed from tyrosine by crystallising from hot alcohol. When pure it crystallises in pearly plates. When impure it is apt to crystallise in soft spherical masses, which have a slightly radiate structure.

It melts at  $297^{\circ}$  with decomposition. When heated gently in an open tube it sublimes at a temperature below its melting point and emits a characteristic smell of amylamine. It is laevorotatory in aqueous solution, but dextrorotatory in solution in hydrochloric acid.

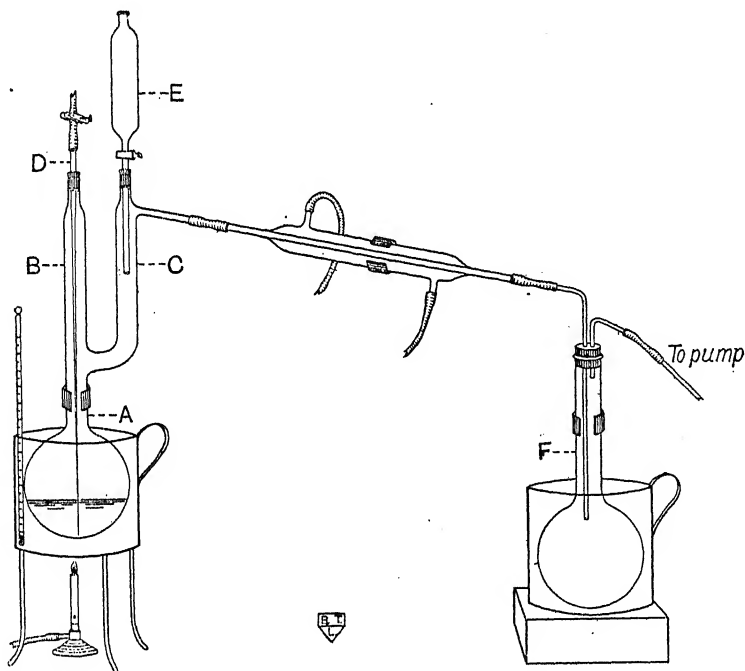


Fig. 13. Distillation *in vacuo* by use of a Claisen flask (A). Alcohol or more of the fluid can be added through E as the distillation proceeds. D is a tube drawn out to a fine capillary through which a small amount of air enters the boiling fluid to prevent excessive bumping.

## CHAPTER V.

### THE CARBOHYDRATES.

There are several groups of these compounds, only a few of which, however, are of any physiological importance.

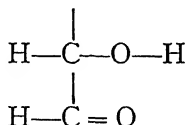
- A. The Monosaccharides, or Simple Sugars.
- B. The Compound Sugars (Di- and Tri-saccharides).
- C. The Polysaccharides.

The first two groups are colourless, crystalline substances, readily soluble in water, and usually of a sweet taste. The polysaccharides are mostly amorphous, insoluble in water, and without a sweet taste.

#### A. The Monosaccharides.

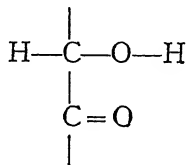
A simple sugar is an aldehyde, or ketone, linked directly to at least one alcoholic group.

Sugars containing the aldehyde group are known as *aldoses*, which therefore contain



as a characteristic group.

Sugars containing the ketone group are known as *ketoses*, of which the group



is characteristic.



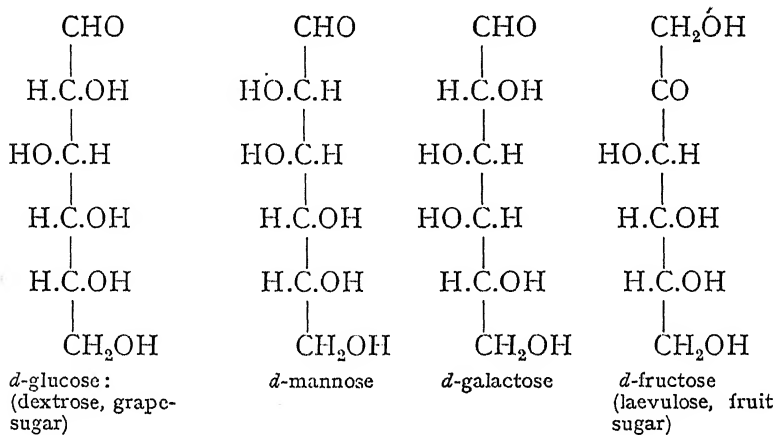
The simple sugars contain from two to nine carbon atoms, and are called *bioses*, *trioses*, *tetroses*, *pentoses*, *hexoses*, etc., depending on the number of carbon atoms they contain.\*

The *pentoses*,  $C_5H_{10}O_5$ , are widely distributed in nature. In plants they are found both in the free state and in the form of condensation products, known as pentosans  $(C_5H_8O_4)_n$ . The most important of the pentoses are the aldoses, arabinose and xylose, best obtained from the corresponding pentosans in gum arabic and beech sawdust respectively.

Ribose is a constituent part of the molecule of yeast nucleic acid.

Pentoses are occasionally found in human urine (see p. 312).

The *hexoses*,  $C_6H_{12}O_6$ , are of great physiological importance. Of the many that have been synthesised in the laboratory only the following are found in nature, and are of physiological interest :—



\* This is not strictly true, for there exist substituted sugars in which one or more H atom is replaced by a methyl group. A methyl pentose thus contains six carbon atoms.





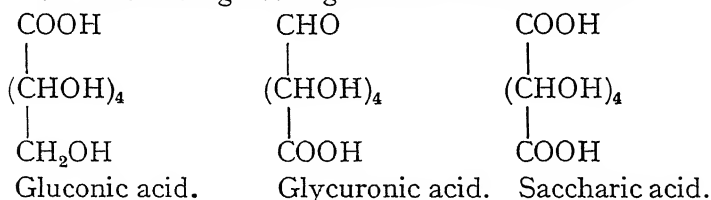
susceptible of being oxidised to various acids, thus reducing certain oxidising reagents. This reaction only takes place in hot alkaline solutions, and is of great value as a test for these sugars, and especially as a basis of various methods of estimation.

They react with phenyl hydrazine in excess to give insoluble crystalline bodies called osazones. These are of the greatest value in determining the presence of and in characterising the monosaccharides, though not in distinguishing them from one another.

When heated with an alkali the monosaccharides become yellow and then brown, and finally decompose into a mixture of acids and resinous substances.

They are reduced by sodium amalgam to hexahydric alcohols. Sorbite is formed from glucose, mannite from mannose and dulcite from galactose. Fructose yields a mixture of sorbite and mannite. These alcohols are of considerable interest, as they are used by bacteriologists for the differential diagnosis of certain pathogenic organisms.

On oxidation glucose gives rise to three acids—



Glycuronic acid is interesting physiologically, as it is frequently found in the urine in combination with various drugs, such as chloral, camphor, phenol, etc. These compounds protect the organism from the injurious effects of the drugs.

On oxidation galactose gives inactive mucic acid, which is isomeric with saccharic acid. Being only slightly soluble its production is used as a test for the presence of lactose in urine, since lactose is hydrolysed by acids into galactose and glucose.

**Glucose** (dextrose or grape sugar)  $C_6H_{12}O_6$ .

**94. Preparation of glucose from starch.**

To 700 cc. of distilled water add 40 cc. of pure HCl and boil. Mix 100 grams. of potato starch with 200 cc. of cold water, and slowly stir this into the boiling mixture. Wash in the remainder of the starch with another 100 cc. of water. Boil under a reflux condenser for 3 hours. To the hot solution add solid lead carbonate, a little at a time, till effervescence ceases (about 100 grams. are usually required). Cool and filter. Evaporate the filtrate to a thin syrup. Add an equal volume of hot 95 per cent. alcohol. Filter. Evaporate the filtrate to a thick syrup. Treat this with twice its volume of 93 per cent. alcohol and allow the solution to cool slowly. If a syrup falls out of solution on cooling, the alcohol is too strong, and a few drops of water should be added and the solution again heated to redissolve it. When the cooled solution no longer deposits any syrup add a crystal of glucose and set aside to crystallise.

After crystallisation is complete, which may take six or seven days, drain the crystals and dry by spreading them on a porous earthenware plate. To recrystallise dissolve the dried crystals in half their weight of water and add to the resulting syrup twice its volume of boiling 93 per cent. alcohol. Set the alcoholic solution aside to crystallise, and dry the resulting crystals as before.

Unless directions to the contrary are given use a 0.2 per cent. solution of glucose for the following exercises.

**95.** Boil 3 cc. with 1 cc. of 5 per cent. sodium hydroxide. The solution turns yellow.

NOTE.—The yellow colour is due to the formation of caramel (a condensation product) by the hot alkali.

**96.** Treat two or three cc. of 5 per cent. caustic soda with four or five drops of a 1 per cent. solution of copper sulphate. A blue precipitate of cupric hydroxide,  $Cu(OH)_2$  is formed. Add to the mixture an equal bulk of the sugar solution. The precipitate dissolves. Boil the solution for a short time. The blue colour disappears, and is replaced by a yellow or red precipitate of cuprous oxide,  $Cu_2O$  (**Trommer's test**).

NOTES.—1. The amount of copper necessary depends on the percentage of sugar present. If only a small amount of sugar be present a mere disappearance of the blue colour is all that may happen, or possibly the fluid may assume a faint yellowish-red tint. If excess of copper be added, the reduction is obscured by the blue cupric hydrate in solution, or the black precipitate of cupric oxide that is formed on heating this in the alkaline solution. It is always best to add the copper sulphate a few drops at a time, boiling between each addition.

2. The reaction is a type of several that have been introduced for the detection of glucose, all of which depend on the fact that in alkaline solution it has reducing properties when boiled. For this reason, glucose, and all sugars that have this property are sometimes spoken of as "reducing sugars."

3. The property that glucose and other sugars have of dissolving cupric hydrate is common to a large number of organic compounds, such as glycerol, Rochelle salt and sodium citrate.

97. Boil about 3 cc. of Fehling's solution (see Note 1) in a test-tube. No change occurs. Add about 3 cc. of the glucose solution and boil again. A red precipitate of cuprous oxide is formed. (Fehling's test.)

NOTES.—1. Fehling's fluid is prepared as follows :

(a) Dissolve 103.92 grams. of pure copper sulphate in warm distilled water and dilute to one litre.

(b) Dissolve 320 grams. of potassium sodium tartrate (Rochelle salt) in warm water, add a little carbolic acid to prevent the growth of fungi, dilute to exactly a litre and filter.

(c) Dissolve 150 grams. of sodium hydroxide in distilled water and dilute to 1 litre.

For use take exactly equal quantities of *a*, *b*, and *c*, and mix. Though the individual constituents keep indefinitely, the fluid when prepared suffers decomposition, so that a reduction occurs on boiling. For this reason the fluid should be prepared just before use, and must always be tested by boiling before being used.

The fluid is of such a strength that the copper sulphate in 10 cc. is just reduced by 0.05 grams. of dextrose.

2. The addition of the Rochelle salt is for the purpose of dissolving the cupric hydroxide that would otherwise be precipitated by mixing (*a*) and (*c*).

3. The test is much more delicate and certain than Trommer's test, and should always be used in preference to it.

4. If the fluid that is being tested is acid, it should be neutralised.

5. Ammonium salts considerably interfere with Fehling's test owing to the ammonia liberated dissolving the cuprous oxide to a colourless compound. If they are present a little extra alkali should be added, and the mixture boiled for two or three minutes to allow of the evolution of the ammonia.

6. In testing for small amounts of glucose it is advisable to avoid an excess of Fehling's solution, owing to the excess of alkali tending to destroy the glucose before the latter can exert its reducing reaction on the copper. The neutral solution should be made faintly blue with Fehling's solution, and then boiled.

98. To 2 cc. of a 1 per cent. solution add 2 cc. of 40 per cent. sodium hydroxide. Heat to boiling and keep boiling for one and a half minutes. To the hot solution add half its volume of Fehling's solution. No reduction, or only a very slight one, is obtained.

NOTE.—Glucose is completely destroyed by boiling with sodium hydroxide.

99. To 3 cc. of a 1 per cent. solution add a large "knife point" of anhydrous sodium carbonate. Boil for 1 minute, cool under the tap. Add half its volume of Fehling's solution, and allow to stand without boiling. The Fehling's solution is reduced without boiling.

NOTE.—The experiment indicates that by the action of alkalies glucose is converted to a material that will reduce Fehling's solution in the cold. Ex. 98 indicates that this material is destroyed by caustic alkalies. It will be seen later (Ex. 118) that the disaccharides, lactose and maltose, differ from glucose in that they reduce Fehling's in the cold after being boiled with either sodium hydroxide or sodium carbonate.

100. To 5 cc. of Benedict's solution in a test-tube, add about eight drops of the sugar solution. Boil vigorously for one or two minutes and allow the tube to cool spontaneously. The entire body of solution will be filled with a precipitate, red, yellow, or green in colour depending on the concentration of the sugar. (**Benedict's test.**)

NOTES.—1. Preparation of Benedict's solution for qualitative test. Dissolve 173 grams. of sodium citrate and 90 grams. of anhydrous sodium carbonate in about 600 cc. of distilled water by the aid of heat. Pour through a folded filter and make up to 850 cc. Dissolve 17.3 grams. of crystallised copper sulphate in 100 cc. of water and make up to 150 cc. Pour the carbonate citrate solution into a large beaker and add the copper solution slowly, with constant stirring. The mixed solution is ready for use and does not deteriorate on long standing.

2. Benedict's solution has certain advantages over Fehling's. For example, it is not so readily reduced by uric acid or urates, nor by creatinine. It is not reduced by chloroform, which is sometimes added to urine as a preservative. It does not destroy a small amount of sugar, as Fehling's does (see note 6 to Ex. 97). Also it can be used for testing urines for sugar in artificial light, since it is the bulk and not the colour of the precipitate that is of importance.

3. Though Benedict's test is much better than Fehling's for the detection of small amounts of glucose in urine, it is not quite so useful for other work. The author claims that his test (Ex. 104) is the most sensitive for general use.

101. To 5 cc. of the modified Barfoed's reagent in a test-tube add 1 cc. of the 0.2 per cent. solution of glucose and stand the tube in a beaker of boiling water. After three and a half minutes remove the tube and examine it against a black background. A definite reduction is obtained. Repeat the experiment with 1 cc. of the

solution diluted 1 in 5. A reduction may or may not be obtained, depending on the sensitiveness of the reagent (**Barfoed's test**, Hinkel and Sherman's modification).

NOTES.—1. The reagent is prepared by dissolving 45 grams. of neutral crystallised cupric acetate in 900 cc. of distilled water and filtering if necessary. To the filtrate add 1.2 cc. of 50 per cent. acetic acid and dilute to 1 litre.

2. A portion must show no change when heated in a boiling water bath for 10 minutes.

3. 0.0005 gram. glucose generally gives the test, whereas 0.02 gram. lactose or maltose, or 0.03 gram. sucrose fail to give the test.

102. Measure 2 cc. of a 1 per cent. solution of glucose into a test-tube. Add 3 drops of pure glycerol. Measure 20 drops of a 20 per cent. solution of pure crystalline copper sulphate into the tube by means of a dropping pipette (see fig. 5). Add 2 cc. of 20 per cent. sodium hydroxide. Boil the mixture and keep it boiling for half a minute, shaking the tube during the boiling to prevent loss by spurting. The addition of a couple of glass beads helps smooth boiling. Filter through a small paper or allow the tube to stand in a rack till the cuprous oxide has settled. Repeat the experiment, using 21, 22, etc., drops of the copper sulphate if the filtrate was yellow; 19, 18, etc., if it was blue, until a point is found at which an extra drop of copper causes a change in the filtrate from yellow to a faint blue.

NOTE.—The experiment gives one a very rough method of determining the concentration of a solution of glucose, which can be applied for finding the approximate dilution necessary when an accurate estimation has to be made. The reason for the addition of glycerol is explained in Ex. 96, note 3.

103. Measure 2 cc. of the 1 per cent. solution into a test-tube, add 0.5 cc. of pure concentrated hydrochloric acid, and boil *gently* for 2 minutes. Cool under the tap. Add the number of drops of copper sulphate necessary to give a faint blue, as found in the preceding exercise, and three drops of glycerol. Neutralise by the addition of 20 per cent. sodium hydroxide, the neutral point being shown by the appearance of a grey precipitate. Now add 2 cc. of 20 per cent. sodium hydroxide, boil for 1 minute, and allow to stand. An increase in the reducing power is not obtained.

NOTE.—Compare the results with those from maltose and lactose, Exs. 120 and 126.

104. To 5 cc. of the solution in a test-tube add a large "knife point" (half a gram.) of anhydrous sodium carbonate. Shake, and



heat to boiling. Maintain active boiling for 50 secs., shaking from side to side to prevent spurting. Immediately add 4 drops of a mixture of equal parts of glycerol and 10 per cent. copper sulphate. Shake for a moment to mix and allow to stand without further heating for 1 minute. The blue colour is discharged, and a yellowish precipitate of cuprous hydroxide forms.

A control test with 5 cc. of distilled water should be performed.

Repeat the experiment, using 5 cc. of the solution diluted 1 in 10 and 1 in 100. (**Cole's test.**)

NOTE.—The test was elaborated by the author for the detection of very small quantities of glucose in urine (see Ex. 381). It is very sensitive, and it is claimed that 1 part of glucose in 500,000 parts of distilled water can be detected by this means. The instructions given are to be strictly followed. Many samples of glycerol give a slight reduction when boiled with sodium carbonate and copper sulphate, but they do not give a reduction when treated in the way described. The function of the glycerol is to keep the cupric carbonate in solution.

105. To 2 cc. of Nylander's reagent add 10 cc. of the glucose solution, mix, and boil. Immerse the tube in a beaker of boiling water for five minutes. A black precipitate of metallic bismuth separates out. (**Nylander's test.**)

NOTES.—1. Nylander's reagent is prepared by dissolving 50 grams. of Rochelle salt and 20 grams. of bismuth subnitrate in 1 litre of 8 per cent. sodium hydroxide.

2. The test is used for detecting small amounts of glucose in urine. It is superior for this purpose to Fehling's solution since it is not readily reduced by urates, creatinine, etc. The introduction of Benedict's solution and Cole's test have, however, led to the disuse of Nylander's.

106. Treat 2 cc. of a 0.1 per cent. solution of safranin with 2 cc. of the glucose solution and 2 cc. of 5 per cent. sodium hydroxide. Mix and boil, avoiding any shaking. The opaque red colour gives place to a light yellow, owing to the reduction of the safranin to a "leuco-base."

107. To 3 cc. of the 0.2 per cent. glucose add 1 cc. of a solution of sulphindigotate of soda and a large "knife point" of anhydrous sodium carbonate and boil. The blue colour turns green, purplish, red, and finally yellow. Shake with air: the blue colour reappears. (**Mulder's test.**)

NOTE.—These two experiments illustrate the reducing properties of glucose in hot alkaline solution. The avidity of the reduced leuco-bases for oxygen is shown by the reappearance of the colour on cooling and shaking with air.

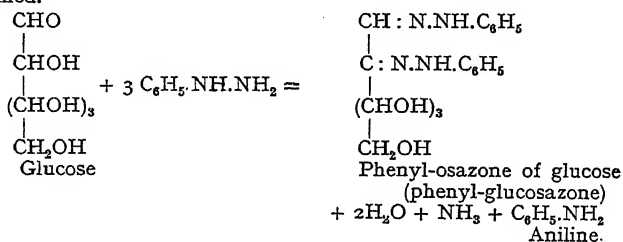
108. To 2 cc. of the solution add a large "knife point" of anhydrous sodium carbonate and a rather smaller amount of solid picric acid. Boil for about a minute. A deep reddish brown colour is produced. Repeat the experiment with 2 cc. of the solution diluted 1 in 10. A distinct colouration is produced.

NOTE.—Picric acid,  $\text{C}_6\text{H}_2 \begin{matrix} \diagup (\text{NO}_2)_3 \\ \diagdown \text{OH} \end{matrix}$  is reduced to picramic acid,  $\text{C}_6\text{H}_2 \begin{matrix} \diagup (\text{NO}_2)_2 \\ \diagdown \text{NH}_2 \\ \text{OH} \end{matrix}$ .

by various substances in alkaline solution. The reaction serves as a basis for the colorimetric method of estimation of sugar in blood (Ex. 311). Note that glucose only gives the test on heating. Creatinine reduces picric acid to picramic acid in the cold (see Ex. 362).

109. To 10 cc. of the solution add 1 cc. of strong acetic acid. Add as much solid phenyl-hydrazine hydrochloride as will lie on a sixpenny piece, and at least twice this amount of solid sodium acetate. Dissolve by warming, mix thoroughly, and filter into a clean tube. Place this in a beaker of boiling water for 30 minutes, keeping the water boiling the whole time. Remove the flame from under the beaker, and allow the solution to cool slowly. A yellow crystalline precipitate of **phenyl-glucosazone** appears, often before the solution has been heated for more than 20 minutes. Collect some of this by means of a pipette, transfer to a slide, cover with a slip, and examine under both powers of the microscope. Note the characteristic arrangement of the fine yellow needles in fan-shaped aggregates, sheaves, or crosses.

NOTES.—1. Glucose is an aldehyde, and, like all aldehydes and ketones, forms a compound with phenyl-hydrazine. But this phenyl-hydrazone of glucose is very soluble, and cannot be readily separated. However, in the presence of an excess of phenyl-hydrazine at 100° C. an insoluble osazone is formed.



2. Phenyl-hydrazine is a yellow basic liquid, insoluble in water, but soluble in dilute acids to form salts. If the base itself is used, two or three

drops should be dissolved in a few cc. of strong acetic acid, and added to the sugar solution.

3. Phenyl-hydrazine hydrochloride,  $C_6H_5.NH.NH_2.HCl$  does not give an osazone when boiled with glucose, unless an excess of sodium acetate be added. This acts on the hydrochloride to form phenyl-hydrazine acetate and sodium chloride. In the author's experience it is advisable to have some free acetic acid present.

4. The osazone can be recrystallised as follows: Filter the cold solution through a small paper. Wash well with cold water. Boil a little strong alcohol in a tube and pour the hot solution on to the paper. Collect the filtrate in a clean tube, boil it, and pass it back through the paper. Repeat the process until a strong alcoholic solution is obtained. Heat it again, and gradually add boiling water until a faint turbidity is produced. Heat again, add alcohol until the solution is just clear again, and then allow the tube to cool slowly. Or the alcoholic solution can be concentrated slightly on a boiling water bath. The product obtained can be filtered, washed and dried in a steam oven. The melting point is  $204^{\circ}$  to  $205^{\circ}C$ .

110. To about 2 cc. of the solution add 6 drops of a 1 per cent. alcoholic solution of  $\alpha$ -naphthol. To this mixture add about 2 cc. of strong sulphuric acid, running it down the side of the tube. A purple ring is formed at the junction of the fluids.

NOTE.—This reaction is given by all carbohydrates (see Ex. 26). Furfural is formed very readily from fructose, sucrose and the pentoses. A modification of the test that only succeeds with these sugars is given in Ex. 114.

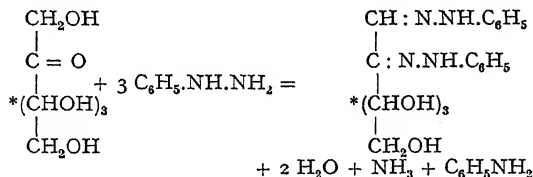
**Fructose** (laevulose or fruit-sugar) is a keto-hexose. It can be prepared by the acid hydrolysis of inulin, a polysaccharide found in the tuberous roots of the dahlia, dandelion, Jerusalem artichoke and *Inula Helenium*, from which plant the name inulin is derived. It can also be prepared by hydrolysing cane sugar with dilute acid and separating the fructose from the glucose by adding calcium hydroxide to the cooled solution. Calcium fructosate crystallises out, and can be decomposed by oxalic acid. It is rather difficult to obtain crystals of the sugar.

For the following reactions use a dilute solution of commercial fructose. Certain of the reactions can be demonstrated by the use of a 1 per cent. solution of "invert sugar," obtained by boiling 100 cc. of 1 per cent. cane sugar with 1 cc. of strong hydrochloric acid for two minutes.

111. Repeat Exercises 95 to 97. They are all obtained.

112. Prepare the osazone as directed in Ex. 109. It is identical with glucosazone.

NOTE.—The reaction between fructose and an excess of phenyl-hydrazine is as follows:—



The configuration of the three secondary alcoholic groups indicated by \* is identical in glucose and fructose (see formula on page 101). It follows that the osazones are identical.

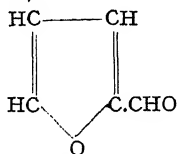
113. Repeat Ex. 110. It is obtained with great brilliance.

114. To about 15 drops in a test-tube add 6 drops of a 1 per cent. alcoholic solution of  $\alpha$ -naphthol and 5 cc. of concentrated HCl. Boil. The solution becomes deep purple as soon as the mixture is vigorously boiling.

NOTES.—1. This modification of the furfural test is given only by fructose, sucrose and the pentoses. Glucose, maltose, lactose, and the common polysaccharides only give an intense colour after being boiled from 1 to 2 minutes.

2. Fructose, either in the free state or produced from sucrose by acid hydrolysis and the pentoses, readily yield furfural (furfuraldehyde) by the action of strong HCl. With  $\text{H}_2\text{SO}_4$  furfural is readily produced from all carbohydrates (see Ex. 110).

3. Furfural is



It reacts with  $\alpha$ -naphthol, thymol, bile salts (see Ex. 315) in the presence of strong acids to give coloured compounds.

4. Proteins that contain a carbohydrate group also give a reaction (see Ex. 26).

115. **Seliwanoff's test** for fructose. To 5 cc. of Seliwanoff's reagent add a few drops of the sugar and heat the solution to boiling. A red colouration and a red precipitate are formed. The precipitate dissolves in alcohol, to which it imparts a striking red colour.

NOTES.—The reagent is prepared by dissolving 0.05 gram. of resorcin in 100 cc. of hydrochloric acid, diluted with its own volume of water.

The test is also given by glucose after long boiling, but a precipitate is not usually formed.]

## B. The Disaccharides.

**Maltose** is the disaccharide formed as the final product of the hydrolysis of starch by enzymes, such as ptyalin, diastase, etc. It is hydrolysed by boiling acids, and by the enzyme maltase of the small intestine, to two molecules of glucose. It exhibits well-marked reducing properties towards Fehling's solution, but not towards Barfoed's. It forms an osazone with phenyl-hydrazine acetate, which is more soluble than glucosazone and which melts at 206°C. Constitutionally it is glucose- $\alpha$ -glucoside.

### 116. Preparation of Maltose.

Weigh out 200 grams. of fine potato starch and divide it into three approximately equal portions. Add 50 cc. of cold water to one portion and stir until a uniform cream is obtained. Pour this slowly into 1200 cc. of boiling water contained in an enamelled iron vessel, stirring well during addition. Boil for a minute, stirring all the time. Cool to 55 C. and add 2 cc. of a fresh malt extract (see note 1).

The starch paste becomes liquified in a few minutes. Boil the liquid again, and to it add the second portion of starch, which has been stirred up with another 50 cc. of cold water. Cool to 55 C., add 2 cc. of malt extract, and liquify as before. Boil again, add the third portion of starch, and cool to 55 C. Add 40 cc. of malt extract, and digest for 24 hours. Boil, filter, and evaporate in a porcelain dish on a water bath until a fairly thick skim forms on the surface. On another water bath heat 500 cc. of 95 per cent. alcohol in a flask and pour it on to the hot syrupy solution, stirring well. The maltose dissolves and the dextrin is precipitated, carrying down with it a considerable percentage of the maltose. Connect the flask to a reflux condenser (fig. 7) and heat on a boiling water bath for 5 hours, repeatedly agitating the mixture. Allow the mixture to cool thoroughly, and pour off the alcoholic solution of maltose from the gummy residue of dextrine. Transfer the alcoholic solution to a distilling flask connected with a condenser and distil off the alcohol as completely as possible by heating the flask on a water bath. Pour the thin syrupy residue

into a beaker and allow to cool. Add a little crystalline maltose and allow to stand for 24 hours in a cool place. The syrup should set to a semi-solid crystalline mass. Spread this on a porous earthenware plate to dry.

*Recrystallisation.*

Weigh the solid, add one-fourth of its volume of water, and heat on the water bath until a syrup is formed. For every cc. of water taken add 10 cc. of hot 88 per cent. alcohol. Filter. Cool, add a little crystalline maltose, and allow to stand for about 2 days. Filter on the pump, wash with a little 95 per cent. alcohol, and dry on a porous plate.

NOTES.—1. Preparation of malt extract. Mix 40 grams. of finely ground pale dried malt with 100 cc. of cold water. Shake well, and allow to stand for four hours. Filter.

2. It is easier to prepare a strong solution of starch by using soluble starch (see p. 395). In this case boil 1200 cc. of water, stir 200 grams. of the soluble starch with 200 cc. of cold water, and pour this slowly into the water, kept hot on a boiling water bath. Cool to 55° C. and add 40 cc. of the malt extract.

Use a 1 per cent. solution for the following exercises:—

117. Repeat Exs. 95, 99 and 100. The reactions are indistinguishable from those of glucose.

118. Repeat Exercise 98. A reduction is generally obtained. (**Distinction from glucose.**)

119. Repeat Exercise 102, using 12 drops of the 20 per cent. copper sulphate for the first trial. It will be seen that the maltose has a reducing power of about 60 per cent. that of a glucose solution of the same strength.

120. Repeat Exercise 103, using 20 drops of the copper sulphate for the first trial. The reducing power of the solution has been markedly increased, owing to the hydrolysis of the maltose to glucose. (**Distinction from glucose.**)

121. Repeat Exercise 101. A reduction is not usually obtained. (**Distinction from glucose.**)

NOTE.—It must be remembered that the glucose solution employed was 0.2 per cent. Two cc. of this can only reduce 4 to 5 drops of 20 per cent. copper sulphate (see Ex. 102). So that, though the 1 per cent. maltose solution employed exhibits strong reducing powers towards alkaline copper

solutions, it has, relatively, a very feeble reducing power towards the acid Barfoed's reagent. It must be emphasised that useful information can only be derived from Barfoed's test if the reducing power of the solution towards alkaline reagents is known.

122. Prepare the osazone as directed in Exercise 109. Malt-osazone is much more soluble than glucosazone, and only separates on cooling. It is important to allow the solution to cool slowly as directed. It generally crystallises in clusters of broad plates, not in needles. It can be recrystallised by dissolving the precipitate in boiling water, filtering, and allowing the hot solution to cool slowly. It melts at  $206^{\circ}\text{C}$ .

**Lactose** is the sugar found in milk, and often in the urine of women during lactation. It has reactions very similar to those of maltose. It is hydrolysed by boiling acids, and by the ferment lactase into equal parts of glucose and galactose.

Constitutionally it is glucose- $\beta$ -galactoside.

It is not fermented by ordinary yeast.

Use a 1 per cent. solution for the following exercises:—

123. Repeat Exs. 95, 97, 99 and 100. The reactions are indistinguishable from those of glucose.

124. Repeat Ex. 98. A reduction is generally obtained.  
**(Distinction from glucose.)**

125. Repeat Ex. 102, using 14 drops of the 20 per cent. copper sulphate for the first trial. Lactose has a reducing power about 70 per cent. of that of glucose.

126. Repeat Ex. 103, using 18 drops of the copper sulphate for the first tube. The reducing power of the solution has been markedly increased, owing to the hydrolysis of the lactose to glucose and galactose. **(Distinction from glucose.)**

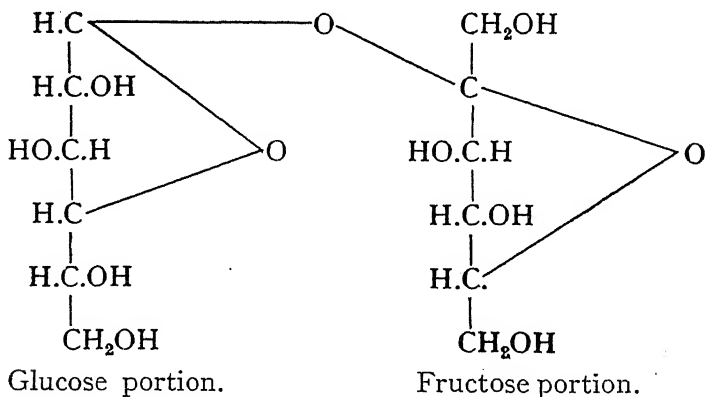
127. Repeat Ex. 101. A reduction is not usually obtained.  
**(Distinction from glucose.)**

128. Prepare the osazone (see Ex. 108). Lactosazone is much more soluble in hot water than glucosazone. It crystallises in irregular clusters of fine needles ("Hedge-hog crystals"). It can be recrystallised from hot water (see Ex. 122). It melts at  $200^{\circ}\text{C}$ .

**Sucrose** (cane sugar) is widely distributed in the vegetable kingdom, where it functions as a reserve material. It crystallises well, is very soluble in water, and has a much sweeter taste than glucose.

It does not reduce Fehling's solution, does not form an osazone, and does not behave as an aldehyde or ketone. It is hydrolysed very readily by boiling acids to a mixture of glucose and fructose. Cane sugar is dextrorotatory, but since fructose is more laevorotatory than glucose is dextrorotatory, a mixture of the two in equal parts is laevorotatory. So the sign of rotation being inverted by hydrolysis, the process is known as inversion, and the product as "invert sugar." This hydrolysis is also effected by the enzyme invertase (sucrase), which is found in the small intestine and in certain yeasts.

The constitution of cane-sugar is not yet definitely established, but in all probability it is formed by the condensation of glucose and fructose in such a way as to destroy both the aldehyde and the ketone groups.



Use a freshly prepared 1 per cent. solution of pure white crystalline cane sugar ("coffee sugar") for the following reactions.

129. Repeat Exs. 95 and 97. Sucrose is not affected by alkali, and does not reduce alkaline copper solutions.



130. To 3 cc. of the solution add 1 drop of concentrated HCl. Boil for a few seconds. Cool under the tap. Add about 10 drops of 20 per cent. copper sulphate, 3 drops of glycerol, and about 3 cc. of 20 per cent. sodium hydroxide. Boil. A marked reduction is obtained.

NOTE.—Sucrose is hydrolysed extremely rapidly by acids into glucose and fructose. Though the polysaccharides yield reducing sugars by acid hydrolysis the above procedure would have very little effect on them.

131. Repeat Exs. 114 and 115. Sucrose behaves like fructose.

### C. Polysaccharides.

These compounds are formed by the condensation of an indefinite number of molecules of monosaccharides.

The *pentosans* ( $C_5H_8O_4$ )<sub>n</sub> yield pentoses on hydrolysis.

The *hexosans* ( $C_6H_{10}O_5$ )<sub>n</sub> yield hexoses, generally glucose, on acid hydrolysis. The polysaccharides described below are hexosans.

**Starch** is widely distributed in the vegetable kingdom as a reserve carbohydrate. It occurs in the form of grains in many roots, tubers, seeds, and leaves. The size and shape of the grains are peculiar to each botanical species.

These grains probably consist of at least two substances.

A. "Amylopectin," or "starch cellulose."

B. "Amylose," or "granulose."

Amylopectin forms about 60 per cent. of the grain. It is a mucilaginous substance, which swells up without dissolving in boiling water or in cold sodium hydroxide. It is hydrolysed by acids into glucose. By certain enzymes, called amylases, found in malt, saliva, and the pancreas, it is converted into a mixture of maltose and "stable dextrin," that is only very slowly hydrolysed to maltose and glucose. It does not seem to give a blue colour with iodine.

Amylose is soluble in cold water. It is rapidly and completely converted to maltose by the amylases without leaving a residuum of dextrin. It gives a blue colour with

iodine. The grains are covered with a film of amylopectin, which prevents the amylose from dissolving out in cold water.

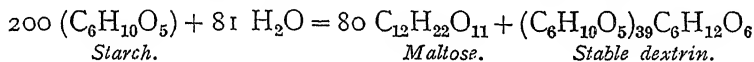
"*Starch paste*" is obtained by pouring a suspension of the grains in cold water into boiling water. It is to be considered as a mixture of amylopectin and amylose, both of the substances being colloids. It is opalescent, due to the amylopectin. It is completely precipitated by half saturation with ammonium sulphate, or by the addition of an equal volume of strong alcohol. It has no reducing properties.

"*Soluble starch*" differs from starch paste in being clear and limpid. It is produced by the action of amylases or acids on starch paste. It is only slowly precipitated by half saturation with ammonium sulphate, but is precipitated immediately by full saturation. It has no reducing properties.

**Dextrins** are formed by the partial hydrolysis of starch by amylases, acids or superheated steam. The name arises from the fact that they are strongly dextrorotatory. They differ considerably in complexity. There are two main varieties: *erythro-dextrins*, giving a reddish colour with iodine, and *achroo-dextrins*, which give no colour. By fractionation with salt solutions Young has separated three erythro-dextrins, I., II., and III. The first two are precipitated by full saturation with ammonium sulphate, and give a purple and a red colour respectively with iodine. Erythro-dextrin III. is not precipitated by salts, and gives a red-brown colour with iodine. The achroo-dextrins also are not precipitated by salts. They are insoluble in strong alcohol and in ether. They reduce Fehling's solution slightly, but do not form osazones nor ferment with yeast.

*Stable dextrin* is the dextrin obtained from starch by the action of amylases continued until the hydrolysis shows an apparent equilibrium. It is, as its name implies, very resistant to the further action of the enzymes, but is apparently broken down slowly to maltose and glucose.

It is possible to regard it as being formed by the condensation of forty molecules of glucose with the elimination of thirty-nine molecules of water. On this assumption its formula would be  $39 (C_6H_{10}O_5) \cdot C_6H_{12}O_6$ . Its reducing power is slight, and has  $[\alpha]_D$  about  $+195^\circ$ . At the stage of apparent equilibrium in the action of amylases on starch, about 85 per cent. of the initial weight is in the form of maltose, and about 19 per cent. of stable dextrin is formed. The increase in weight is due to the addition of the elements of water in the hydrolysis. The following equation has been suggested by Brown and Millar.



*Malto-dextrin* is the name given to an achroo-dextrin obtained by the action of malt diastase in starch. It is rapidly hydrolysed to maltose by the amylases.

The exact relationships between these various dextrins to one another and to the constituents of the starch grain are so imperfectly understood at present that it is not considered advisable to give a scheme of the stages of hydrolysis.

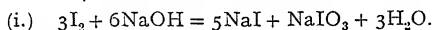
r32. Place a small amount of dry potato-starch on a slide, add a drop of water, cover with a slip, and examine under the microscope. Note the characteristic oval starch grains, the concentric markings and the hilum, usually eccentric. Make a drawing of the grains. Run a drop of iodine under the slip ; note that the grains take on a blue colour.

133. Shake a small amount of potato starch with cold water. The starch does not dissolve. Filter, and add a drop of iodine solution to the filtrate. The characteristic blue reaction is not obtained.

r34. Shake some dry starch with a little sodium carbonate solution. No change is effected. Shake another portion of starch with a little sodiumhydroxide. The starch is immediately gelatinised. To this jelly add a few drops of iodine solution: a blue

colour is not obtained. Treat with strong acetic acid. A deep blue colour appears.

NOTE.—Free iodine is necessary to give the blue adsorption compound with starch. Sodium hydroxide removes free iodine, converting it into iodide and iodate. The action of the acid on the latter causes the appearance of free iodine and the blue colour. *Always neutralise an alkaline solution before testing for the polysaccharides.* The following equations show the effect of sodium hydroxide on iodine, and of acid on a mixture of iodide and iodate:



135. **Preparation of starch paste.** Boil about 75 cc. of distilled water in a beaker. Weigh out 1 gram. of dry potato starch in another small beaker, add about 10 cc. of cold water, and stir to get a uniform suspension. Pour this into the boiling water and stir well. Wash the small beaker out with another 10 cc. of cold water, adding this to the boiling fluid. Stir again, and keep boiling for 1 minute. Cool, and make the volume up to 100 cc. Note that the "solution" is distinctly opalescent. It should be quite uniform and free from lumps.

136. To a small amount of the paste add a drop or two of dilute iodine. A deep blue colour is produced.

NOTE.—The iodine solution should be about 0.01 N. (See appendix, p. 390.)

137. Treat 5 cc. of the cold starch paste with an equal bulk of saturated ammonium sulphate. Shake the test-tube and allow it to stand for five minutes. The starch is precipitated. Filter through a dry paper, and add a drop of iodine solution to the filtrate. No blue colour, or only the very slightest tint is obtained, showing that the whole of the starch paste is precipitated by half-saturation with ammonium sulphate.

138. Boil 5 cc. of the starch paste with two drops of concentrated sulphuric acid for about 15 seconds. Note that the solution becomes perfectly clear and translucent. Add two drops of strong ammonia to neutralise the acid, cool under the tap, add an exactly equal bulk of saturated ammonium sulphate, shake the tube vigorously, and allow it to stand for five minutes. Filter through a dry filter-paper and add two drops of iodine solution to the filtrate. A deep blue colour is obtained.

NOTE.—Starch paste is rapidly converted into “soluble starch” on boiling with dilute mineral acids. Soluble starch differs from starch paste in that it is not completely precipitated by half-saturation with  $(\text{NH}_4)_2\text{SO}_4$  in the course of five minutes. If it be allowed to stand for twenty-four hours, however, it is completely precipitated.

139. Measure 2 cc. of the paste into a test-tube, add 6 drops of concentrated hydrochloric acid by means of a dropping pipette (see fig. 5). Heat to boiling and maintain the boiling for one minute by the watch. Cool thoroughly under the tap. Add first one drop, and then another drop, of the iodine solution. A red or violet colour is produced, indicating the conversion of the starch into crythro-dextrin by acid hydrolysis.

140. Boil 2 cc. of the paste with 6 drops of concentrated hydrochloric acid as before. Cool. Add 3 drops of glycerol and 8 drops of 20 per cent. copper sulphate. Add 20 per cent. sodium hydroxide until a grey precipitate of basic copper sulphate is produced. Now add another 2 cc. of the sodium hydroxide and boil for a minute. A slight reduction is usually obtained.

141. Repeat the previous exercise, using 20 drops of the acid, and boiling for two minutes. Cool. Add 3 drops of glycerol and 16 drops of the copper sulphate. Neutralise with soda and then add 2 cc. in excess. Boil for one minute. Complete, or nearly complete, reduction of the copper is obtained, indicating that the starch has been hydrolysed to glucose (see Ex. 102).

NOTE.—If 12 drops of hydrochloric acid be added and the mixture boiled for one minute, it will generally be found that only a yellow colour is produced with iodine, and that the amount of glucose formed is not sufficient to reduce 9 drops of copper sulphate. At this stage a considerable proportion of the carbohydrate is in the form of achroo-dextrin. It is important to note that the complete hydrolysis of starch by acids is relatively slow compared to that of sucrose and the other disaccharides (see Ex. 130). The addition of a couple of glass beads makes it easier to obtain smooth boiling in the above exercises.

142. Shake a little commercial dextrin with some cold water. An opalescent solution is formed. Boil the solution. It becomes perfectly translucent. (Distinction from glycogen.)

Use a 3 per cent. solution of commercial dextrin for the following reactions:—

143. To about 5 cc. of the dextrin solution add iodine solution, drop by drop, noting the colour at every addition. The colour is at

149. Take 10 cc. of the dextrin solution in a small flask; add 30 cc. of strong (95 per cent.) alcohol, place the thumb over the mouth of the flask and shake vigorously for some seconds. Note that a portion of the dextrin is precipitated as a gummy mass which sticks to the sides of the flask.

Pour off the alcohol, filter it and label the filtrate A. Rinse the flask out with a few cc. of alcohol, shake off as much of this alcohol as possible, and add 10 cc. of hot water. Shake this round the flask till the whole of the gummy precipitate dissolves. Divide the solution into three portions, B, C, and D. To B add a drop of iodine: a purple colour is produced. Boil C with a little Fehling's solution: only a slight reduction takes place. Boil D with two drops of concentrated sulphuric acid for two minutes, neutralise with sodium hydroxide, and boil with a little Fehling's solution: a well-marked reduction occurs.

150. To a portion of filtrate A, add a drop of iodine solution. No colour is produced. To another portion of about 5 cc. add an equal bulk of strong alcohol. A white precipitate of achroo-dextrin is formed.

**Glycogen** is a reserve polysaccharide found in the liver and muscles. It forms a white amorphous powder, soluble in water to form an opalescent solution. It is precipitated from solution by the addition of an equal volume of strong alcohol or by full saturation with ammonium sulphate. It does not reduce Fehling's solution, form an osazone nor ferment with yeast. It gives a reddish colour with iodine. By boiling acids it is hydrolysed to glucose: by most of the diastatic enzymes to maltose, but by the diastase found in the liver to glucose. It is not affected by boiling alkalis. It is dextro-rotatory.

**Estimation.** Pflüger's method is undoubtedly the best. 20 to 100 grams. of the tissue is cut into small pieces and placed in an Erlenmeyer flask of Jena glass. 100 cc. of 60 per cent. potash ("pure by alcohol"—sp. gr. 1.438) is added, a reflux condenser fitted, and the flask immersed for three hours in a boiling water bath. The alkali destroys the proteins without attacking the glycogen.

After cooling 200 cc. of water and 800 cc. of 96 per cent. alcohol are added, and the mixture left to stand over-night. The glycogen is thus precipitated free from protein. The supernatant fluid is carefully decanted and filtered. The precipitate is washed with ten times its volume of 66 per cent. alcohol, containing 1 cc. per litre of saturated sodium chloride. After settling, the

first almost a pure blue, but it changes through a rich purple-red to a red-brown as the iodine is added.

NOTE.—Some samples of commercial dextrin contain a considerable amount of soluble starch.

144. Repeat the above experiment, but boil and then cool the tube after each addition. The colour disappears on boiling, but does not reappear on cooling until several drops of iodine have been added, unless much soluble starch is present.

145. Add a drop or two of the starch paste prepared in Ex. 135 to about 5 cc. of the dextrin solution. To this mixture add diluted iodine solution, drop by drop. The first additions produce a pure blue colour, and it is not till a considerable amount of iodine has been added that the solution acquires a purplish tint.

NOTE.—The affinity of starch for iodine is so much greater than that of dextrin that the characteristic colour reactions of erythro-dextrin are not obtained until all the starch has been saturated with iodine. Even then it is sometimes difficult to detect, owing to the deep blue starch reaction.

146. Treat 5 cc. of the dextrin solution with about 10 drops of the starch paste: to the mixture add an equal bulk of saturated ammonium sulphate, shake vigorously, and allow to stand for five minutes. The starch is precipitated. Filter through a dry paper, and to a portion of the filtrate add a drop or two of iodine solution. The purple red reaction of erythro-dextrin is obtained.

147. Saturate 5 cc. of the dextrin solution by boiling with an excess of finely powdered ammonium sulphate. Note the precipitate of erythro-dextrin produced. Cool under the tap and filter. To the filtrate add a drop of iodine. A red-brown colour is produced.

NOTE.—This colour is due to the fact that erythro-dextrin III. is not precipitated by ammonium sulphate. This is the method employed for the identification of erythro-dextrin in the presence of glycogen, which is completely precipitated by saturation with ammonium sulphate.

148. Boil a few cc. of the dextrin solution with a small amount of Fehling's fluid. A well-marked reduction is usually obtained.

NOTE.—Commercial dextrin is generally prepared by the action of dilute acids on starch (see Ex. 139), the action being stopped as soon as a portion fails to give a blue colour with iodine, and the products then being precipitated by alcohol. Such preparations contain some glucose, and often a little soluble starch. At the same time it must be noted that the achroo-dextrins have a reducing action themselves even when thoroughly separated from the glucose.

fluid is filtered through the original filter paper. This process is repeated once more, and then the precipitate is shaken with ten times its volume of 96 per cent. alcohol and filtered through the same paper. The precipitate is washed with ether, dissolved in boiling water and the solution made up to one litre. 200 cc. of this are treated with 10 cc. of concentrated HCl and heated in a flask on a boiling water bath for three hours, to convert the glycogen into glucose. After cooling, the solution is neutralised with 20 per cent. potash and filtered through a small paper into a 250 cc. measuring flask. The washings from the flask used for inversion are filtered through the same paper to remove the last traces of glucose, and the solution brought up to 250 cc. The percentage of glucose in the solution is determined by analysis. This multiplied by .927 gives the amount of glycogen in the 200 cc. of the solution used for inversion, and so the percentage in the tissue can be readily calculated.

**Preparation.** A rabbit, which has had a full meal of carrots some five or six hours previously, is killed by decapitation. The liver is cut out as quickly as possible, and the gall-bladder removed. The liver is rapidly chopped into small pieces, a small portion being reserved for Ex. 156, and the remainder immediately thrown into boiling water. After about two minutes boiling the larger morsels are strained off, pounded to a paste with sand in a mortar, and replaced in the boiling water. The proteins in solution are then coagulated by making the boiling fluid just acid with acetic acid. The fluid is filtered through coarse filter paper. In this way a crude solution of glycogen is obtained.

151. Boil 5 cc. in a test-tube. The characteristic opalescence does not disappear. (Distinction from erythro-dextrin.)

152. To a small amount of the cooled solution add iodine, drop by drop. A red colour is formed, which disappears on shaking, until with a certain amount of iodine added it is permanent. Now heat the solution. The colour disappears, to reappear on cooling.

NOTE.—If much protein is present in solution the colour will not reappear on cooling unless a considerable amount of iodine be added. This is due to the fact that proteins combine with iodine to form an iodo-protein.

153. Saturate 10 cc. of the solution by boiling with an excess of finely-powdered ammonium sulphate; cool thoroughly under the tap. The glycogen is precipitated. Filter, passing the filtrate again through the paper if it comes through cloudy. Add a drop or two of iodine to the filtrate. No red colour is produced. Scrape the precipitate off the paper, boil with a small amount of water. The solution is markedly opalescent. Cool the solution and add iodine. A port-wine red colour is obtained.

154. Boil 5 cc. of the solution with a little Fehling's fluid. A very slight or no reduction is obtained.

NOTE.—If the liver has been rapidly boiled, no sugar will be present. If delay has occurred during the initial stages of the preparation; some of the glycogen will have been converted into glucose. (See Ex. 156.)



155. To 10 cc. of the solution add 20 cc. of strong alcohol, shake vigorously and filter. To a portion of the filtrate add iodine solution. No colour is obtained, showing that the whole of the glycogen is precipitated. Dissolve the precipitate in a little hot water: note that it is opalescent. Add three drops of strong sulphuric acid and boil for about three minutes: the opalescence disappears. Neutralise with sodium hydroxide and apply Fehling's test. A marked reduction occurs, due to the conversion of the glycogen into glucose by the boiling acid.

156. The portion of rabbit's liver that was reserved has been kept in a warm place for about six hours and extracted with boiling water as before. (Or a decoction of the liver of a sheep obtained from a butcher may be used.) Note that the solution is almost translucent. To a portion add iodine: only a very slight or no red colour at all is produced. To another portion apply Fehling's test: a well-marked reduction occurs.

157. Prepare a solution which contains equal quantities of 1 per cent. starch paste (freshly prepared), of a strong solution of glycogen and of a 3 per cent. solution of commercial dextrin. Note that the mixture is markedly opalescent.

To a small portion add diluted iodine, and note that a pure blue *starch* reaction is obtained.

To another portion of about 5 cc. add an equal bulk of saturated ammonium sulphate, shake vigorously, leave for five minutes, and filter. Note that a portion of the filtrate gives a reddish colour with iodine, and that it is distinctly opalescent. Indication of the presence of *glycogen*.

Saturate the remainder of the fluid by boiling with finely-powdered ammonium sulphate. Cool and filter. The filtrate gives a reddish-brown colour with iodine. Indication of the presence of *erythro-dextrin*.

#### D. The Quantitative Estimation of the Carbohydrates.

A very large number of methods have been introduced for the estimation of glucose, etc., and considerable

experience is required to select the method best suited for a given purpose.

The following scheme indicates the principle of the more important methods, only a few of which are described below or in other sections of this book.

#### A. Direct Volumetric Methods.

1. *Fehling's*. See p. 141.

2. *Ling's modification*. See p. 141.

3. *Pavy's*. Strong ammonia is added to Fehling's solution. A measured amount of this is boiled and the sugar solution run in from a burette. The cuprous oxide formed is kept in solution by the ammonia (see Ex. 97, note 5), forming a colourless compound. The end point is thus much easier to see. The practical difficulty of the method is to regulate the heating and also the rate at which the sugar solution is added.

4. *Benedict's*. See p. 127.

5. *Folin and McEllroy's*. See p. 129.

#### B. Indirect Volumetric Methods.

6. *Amos Peters'*. See p. 134.

7. *Bertrand's*. The sugar is heated with an excess of Fehling's solution. The cuprous oxide formed is filtered off through asbestos and dissolved in an acid solution of ferric sulphate, some of which is reduced to ferrous sulphate. The amount of the latter is determined by titration with standard permanganate.

8. *Wood-Ost's*. See p. 131. This is very similar to the above, except that copper bicarbonate is used instead of Fehling's.

9. *Cole's micro-method*. See p. 253.

#### C. Colorimetric Methods.

10. *Benedict's*. See p. 251.

### D. Polarimetric Method.

II. This is of great value. The relationships between reducing and rotatory powers of solutions before and after hydrolysis must be determined for the identification and analysis of mixed carbohydrates.

Of the various methods proposed, the Author is of the opinion that for ordinary routine work and for urinary analysis, Benedict's direct volumetric method is the most reliable in the hands of the majority of workers. The recent method of Folin and McEllroy has certain advantages, especially in the cost of materials, and may eventually supersede Benedict's. The Wood-Ost process is worthy of more extended recognition. In spite of the 10 minutes' boiling that is necessary, the method is a rapid one, and when completed one is left with a sense of confidence that is somewhat lacking in the direct methods.

For accurate research work the method of Amos Peters is extremely satisfactory.

When a large series of diabetic urines have to be examined sufficiently approximate results can be obtained by polarisation, after removal of the pigment by blood charcoal in the presence of 10 per cent. of acetic acid, a little freshly prepared metaphosphoric acid being added if proteins are present.

#### 158. Benedict's Method.

*Principle of the Method.*—An alkaline solution of copper sulphate, containing thiocyanate is boiled and the sugar solution run in from a burette till the blue colour just disappears. The thiocyanate forms a white insoluble compound with the cuprous hydroxide formed by the reduction of the copper, and so there is no red cuprous oxide precipitated to obscure the blue tint. A little potassium ferrocyanide is also added to prevent any possibility of the deposition of the cuprous oxide.

*Preparation of the Solution.*—With the aid of heat dissolve

Sodium citrate           ..    ..    ..    200 grams.

Sodium carbonate (cryst.)    ..    200 grams.

(or anhydrous sod. carb. 75 grams.)

Potassium thiocyanate (sulphocyanide)   125 grams.

in enough distilled water to make about 800 cc. of the mixture and filter, and cool to room temperature.

Dissolve 18 grams. of the purest, air-dried crystalline copper sulphate in about 100 cc. of distilled water, and pour it slowly into the other liquid with constant stirring. Add 5 cc. of a 5 per cent. solution of potassium ferrocyanide and then distilled water to make

the total volume 1000 cc. The solution appears to keep indefinitely, without any special precaution, such as exclusion of light, etc.

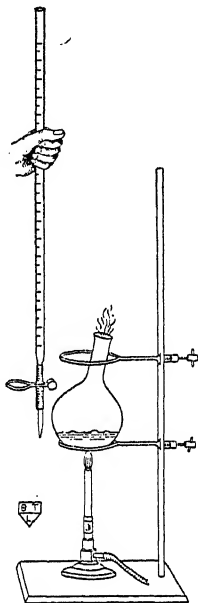


Fig. 13A.

Apparatus for  
Benedict's Method.

*Method of Analysis.*—Fit a 150 cc. flask into a ring of a retort stand of such a size that it is fairly firmly held. There is no need to use a wire gauze. Arrange the flask at such a height over a Bunsen burner that the reagent can be kept briskly boiling by means of a *small* flame. In the flask place 3 to 4 grams. of anhydrous sodium carbonate. This can be roughly measured by taking a depth of 1 inch in a dry test-tube. Then add 25 cc. of the reagent and heat till most of the carbonate is in solution. Run the sugar solution in from a burette, which is best held in the hand. Run the sugar in *slowly*, till a bulky chalk-white precipitate is formed and the blue colour lessens perceptibly in intensity. From this point the sugar is added more and more slowly, with **constant boiling**, until the disappearance of the last trace of blue colour, which marks the end-point. If the volume of the sugar is less than 5 cc., dilute it accurately with water till about 10 cc. are judged necessary.

Repeat the titration with this as before.

**NOTES.**—There is a tendency to run in an excess of the sugar, unless special care is exercised throughout the titration, and particularly at the end. The solution must be kept steadily boiling during the entire process, and towards the end the sugar must be added in portions of a drop or two, with an interval of about 30 seconds after each addition. Should the mixture become too concentrated, boiled distilled water may be added to replace that lost by evaporation.

The titration can also be carried out in a white porcelain dish of 10 to 15 cm. in diameter, but the risk of reoxidation of the cuprous compound is greater than in a flask.

Should the solution bump excessively, two or three small pieces of broken porcelain may be added.

The 3 to 4 grams. of anhydrous sodium carbonate are added to produce the necessary alkalinity. This proportion of alkali cannot be added to the bulk of the standard solution, for it would crystallise out at room temperature.

*Calculation of Results.*

25 cc. of Benedict's solution are reduced by 0.05 gram. of glucose.  
0.053 gram. fructose.  
0.074 gram. maltose.  
0.0676 gram. lactose.

*Example.*—First titration required 2.4 cc.

Solution diluted 1 in 4 (10 cc. of sugar diluted with 30 cc. water).

Second titration required 9.7 cc.

So 9.7 cc. diluted solution contain 0.05 gram. glucose.

100 cc. diluted solution contain  $\frac{0.05 \times 100}{9.7}$

100 cc. original solution contain  $\frac{0.05 \times 100 \times 4}{9.7} = 2.06$  gm.

### 159. The method of Folin and McEllroy.\*

**Principle.** Five cc. of a 6 per cent. solution of crystalline copper sulphate are treated in a large test-tube with a mixture of sodium phosphate and sodium carbonate. A deep blue solution is obtained on boiling. 2 cc. of a strong solution of potassium thiocyanate are now added. The sugar solution is run in from a burette. A white precipitate of cuprous thiocyanate is formed. The sugar is run in very slowly until the blue copper colour is just discharged. Owing to the reduction of the alkalinity of the solution, the amount of copper reduced by a given amount of sugar is considerably greater than in Fehling's or Benedict's methods. Also the reoxidation of the cuprous salts to the cupric condition is relatively very slow. The main objection to the method is the relatively slow rate of reduction.

#### Reagents required :

1. *Copper sulphate.* Dissolve 60 grams. of the best air-dried crystalline copper sulphate in distilled water, add 2 or 3 cc. of pure sulphuric acid, and make the volume up to 1 litre. The acid is to prevent the slow deposition of copper hydroxide and silicate due to the action on the glass.
2. *Alkaline phosphate powder.* Mix together in a large mortar, 100 grams. disodium phosphate crystals ( $\text{HNa}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$ ), and 60 grams. of anhydrous sodium carbonate.
3. To 40 grams. of sodium or potassium thiocyanate add 50 cc. of distilled water. When dissolved make the volume up to 100 cc.

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\* *Journ. of Biological Chemistry*, xxxiii., p. 516 (1918).

4. *Special Burette.* Folin and McElroy describe an ingenious method of measuring small quantities of fluids by means of an accessory line tip fitted to an ordinary burette. When the fluid is dropping from the burette very slowly, the size of the drop is constant for a particular fluid. So if the number of drops emitted by a given tip for a delivery of 1 cc. be known, the volume of the number of drops required for the titration can be readily calculated. The author prefers to use the micro-burette shewn in fig. 14. This may be of 2 or 5 cc. capacity. A rubber tube and spring clip is preferable to a glass tap and accessory tip, as the grease from the tap gets into the burette and makes it impossible to get an accurate reading. As recommended by Folin and McElroy, the burette must be filled by suction. Elaborate washing of the burette in the intervals between successive estimations is thereby rendered unnecessary.



Fig. 14  
Micro-  
burette

**Method.** Weigh out approximately 5 grams. of the phosphate mixture and transfer it to a clean, dry tube, conveniently 7 inches by  $\frac{3}{8}$ ths inch. Add 5 cc. of the 6 per cent. solution of copper sulphate shake and heat to boiling. A deep blue solution is obtained. Add 2 cc. of the strong solution of thiocyanate and heat again. Run in about 0.5 cc. of the sugar solution from the burette and boil *very gently* for 2 minutes by the watch. The tube should be held at an angle and moved about in a *small* flame: excessive concentration and loss by spurting can be thus avoided. If there is an appreciable amount of sugar present a chalky white precipitate appears. If the blue colour entirely disappears there is more than 5 per cent. of sugar present, and the estimation must be repeated with a diluted solution. If the copper is only slightly reduced, yielding only a small amount of cuprous thiocyanate, add a further amount of the sugar solution and boil *gently* for another minute. If now the greater part of the copper has been reduced, complete the titration by adding a drop at a time, boiling for 1 minute after each addition. The total period of boiling must not be less than 4 minutes,

and should not exceed 8. The copper value has been adjusted to a period of 5 to 6 minutes.

A second estimation is often necessary. With a little experience it is easy to judge of the amount that should be added, so that

after a preliminary boiling period of 3 minutes, only a few drops more are required to complete the titration.

With pure glucose solutions the end point is very sharp. With lactose, maltose and diabetic urines the end point is the transition from a green to a yellow colour.

The special precaution necessary is to ensure that the boiling period is within the stated limits.

#### Calculation of results.

|                                    |                             |
|------------------------------------|-----------------------------|
| 5 cc. of the copper are reduced by | 25 mg. glucose.             |
| " " "                              | 25 mg. fructose.            |
| " " "                              | 40.4 mg. anhydrous lactose. |
| " " "                              | 45 mg. anhydrous maltose.   |

In the case of glucose the concentration in grams. per cent. is 2.5 divided by volume of solution required.

#### 160. The Wood-Ost copper carbonate method.\*

*Principle.* A solution of copper sulphate in carbonate and bicarbonate of potash is boiled with a given volume of the sugar solution. The cuprous oxide formed is filtered off through asbestos and washed with cold water. It is suspended in acid ferric sulphate and the amount of ferrous sulphate formed determined by titration with standard potassium permanganate. The amount of copper reduced being known, the weight of sugar in the volume taken can be determined by reference to a curve or tables.

##### *Preparation of solutions.*

1. *Copper carbonate.* Dissolve 250 grams. of potassium carbonate and 100 grams. of potassium bicarbonate by the addition of about 600 cc. of warm distilled water. Dissolve 23.5 grams. of pure crystalline copper sulphate in about 200 cc. of water. Gradually add the copper to the carbonate solution, mixing well during the addition. Make the volume up to 1000 cc. and filter. The solution seems to keep indefinitely.

2. *Acid ferric sulphate.\** Gradually add 250 cc. of pure concentrated sulphuric acid to 750 cc. of distilled water. Add 25 grams. of ferric sulphate. Warm till the sulphate has dissolved, and filter if necessary. The solution must not be used until it has cooled.

3. *Standard potassium permanganate.* Dissolve about 6 grams. of potassium permanganate in a 100 cc. of cold distilled water. Having made certain that the whole has dissolved standardise the solution as follows:—Weigh out between 0.3 and 0.4 gram. of pure crystalline ammonium oxalate, determining

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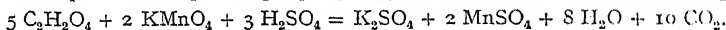
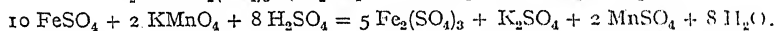
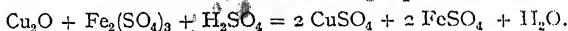
\* T. B. Wood and A. Berry, *Cambridge Philosophical Journal*, xlv., p. 103 (1904).

the exact weight to a milligramme. Add about 50 cc. of distilled water, to which about 3 cc. of *pure* concentrated sulphuric acid have just previously been added. Warm on a water bath until the solid has completely dissolved. Titrate the warm solution with the permanganate. This must be run in very slowly at first, further additions not being made until the colour has completely faded. The end point is reached when a faint rose colour persists for at least a minute. If  $A$  be the weight of ammonium oxalate taken, and  $P$  the volume of permanganate required, then 1 cc. of permanganate corresponds to

$$\frac{895.1 \times A}{P} = T \text{ mg. copper.}$$

It is convenient to have  $T = 10$ . If  $T$  be greater than 10, add 100 ( $T - 10$ ) cc. of water to 1 litre of the solution. If the titration has been conducted accurately, 1 cc. of the permanganate corresponds to 1 mg. copper.

The calculation is based on the following equations:—



So 1 mol. of oxalic acid or of ammonium oxalate ( $\text{C}_2\text{O}_4\text{N}_2\text{H}_8 \cdot \text{H}_2\text{O}$ ) requires the same amount of permanganate as 2 Fe, which corresponds to 2 Cu.

$$\text{So } P \text{ cc. of permanganate} = \frac{2 \times 63.5 \times A}{142.1} = 0.8951 \times A \text{ gm. Cu.}$$

**Method.** Measure 50 cc. of the copper solution into a 150 cc. flask of "Duro" glass. Add two or three small pieces of broken porcelain to prevent subsequent bumping. Boil by heating on a gauze with a Bunsen. As soon as the solution has commenced to boil run in exactly 10 cc. of the sugar solution, which should be between 0.2 and 0.9 per cent. of glucose (see note 1). Note the exact time when the boiling recommences. The flame should be moderately high at first, but as soon as the solution recommences boiling after the addition of the glucose, it should be lowered so that it just maintains *gentle* boiling. After exactly ten minutes' boiling, stop the reduction by immersing the flask in cold water. Then cool thoroughly under the tap.

*Filtration of the cuprous oxide.* This is done through asbestos by means of a Gooch crucible of 25 to 50 cc. capacity (fig. 48), or, better, through an asbestos mat supported on a small (15 mm. diam.) perforated porcelain plate, resting in a conical funnel that passes through a rubber stopper fitting the neck of a filtering flask. The preparation of the mat and the subsequent filtration is most facilitated by use of the special pump connexions shewn in fig. 6, p. 74. The mat is prepared as follows: the suspension of well-washed



asbestos (see note 2) is poured on to the plate (or into the Gooch crucible) and allowed to settle down without suction. After a short time the tube E (fig. 9) is connected to the filtering flask, the tap C being turned so that it connects to B (thus practically preventing suction through E), and the water pressure turned on. The tap C is then turned through an angle so as to allow suction through E, but before the water has been completely drained off, the tap is rapidly opened again, it being important not to suck too hard. The amount of asbestos required is such as to form a mat about 2 mm. in depth. A small porous plate may be placed on the top of the mat to prevent the latter from being washed too much by pouring on water or the copper solution. The solution is then washed two or three times with distilled water, gentle suction being applied after each addition. The final suction should be sufficient to make the mat quite firm. The mat should be prepared during the heating process, it only requiring a few minutes, in spite of this long description. The cold copper solution is poured on to the upper porcelain plate and suction then started. The pressure must be released before all the solution has passed through, it being most important to avoid caking the cuprous oxide by too high a pressure. The flask is washed out with about 10 cc. of cold distilled water (which may, with advantage, have been recently boiled and cooled), and this poured on to the cuprous oxide on the asbestos and filtered through as before. This washing is repeated twice more, care being taken all the time to prevent caking of the cuprous oxide by too high a filtration pressure.

*Solution of the precipitate.* Measure 25 cc. of the ferric sulphate solution by means of a measuring cylinder. Pour about 5 cc. of this into the boiling flask and shake this round to dissolve any cuprous oxide that is sticking to the walls of the vessel. Transfer the filtering mat and the filtering discs to a small beaker by means of a small glass rod that has a pointed hook. Remove the funnel from the filtering flask and wash it down into the beaker with the remainder of the acid ferric sulphate. Wash out the flask two or three times with small quantities of water, transferring this to the beaker through the funnel. Wash down the small rod and stir well with a larger glass rod, which should not be guarded with a rubber collar, since permanganate attacks rubber. The cuprous oxide may all

dissolve, but a certain amount may remain in suspension until the permanganate titration is nearing completion.

*Titration of the reduced iron.* Run in the potassium permanganate from a burette fitted with a glass stopcock, stirring the mixture well. From time to time examine the beaker by holding it above the head. Any lumps of undissolved cuprous oxide can thus be detected. They must be broken up and brought into solution by rubbing with the rod. It is most important that this should be done before the titration is completed. The end point is reached when a faint pink tinge persists for at least ten seconds.

*Calculation of results.* One cc. of permanganate = 10 mg. Cu. The amount of sugar corresponding to various amounts of copper are obtained by plotting the results given below. The amount of sugar corresponding to the exact amount of copper reduced is thus found. The number of milligrammes of sugar in 10 cc. divided by 10 and multiplied by the dilution employed gives the percentage of sugar.

| mg. Cu. | mg. Glucose<br>anhydride | mg. Maltose<br>anhydride | mg. Cu. | mg. Glucose<br>anhydride | mg. Maltose<br>anhydride |
|---------|--------------------------|--------------------------|---------|--------------------------|--------------------------|
| 25      | 7.3                      | 14.0                     | 175     | 53                       | 99                       |
| 50      | 15                       | 28.2                     | 200     | 60.5                     | 113                      |
| 75      | 22.4                     | 42.3                     | 225     | 69.5                     | 130.4                    |
| 100     | 30                       | 56.2                     | 250     | 79.2                     | 147.5                    |
| 125     | 37.8                     | 70.5                     | 275     | 89                       | 164.6                    |
| 150     | 45.3                     | 84.5                     | 290     | 95.4                     | 175                      |

NOTES.—1. A rough approximation of the concentration of glucose in the original solution can be made by use of Fehling's solution. The sugar should be so diluted that 3 to 5 cc. reduce 3 cc. of Fehling's solution.

2. Preparation of the asbestos. (See appendix.)

## 161. The estimation of glucose by the method of Amos Peters.

**Principle.** A known volume of the sugar solution is boiled with a measured amount of an alkaline solution of copper sulphate. The cuprous oxide is filtered off and the copper in the filtrate determined by treatment with potassium iodide and titration of the iodine liberated by

means of a solution of sodium thiosulphate. From the amount of copper reduced the amount of glucose in the volume of solution taken can be determined.

#### Solutions required.

1. Copper sulphate. 69.278 grams. of the purest crystalline salt  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , is dissolved in water and the volume made up to 1 litre.
2. Alkaline tartrate. 346 grams. of Rochelle salt and 250 grams. of pure potassium hydroxide are dissolved in water and the volume made up to 1 litre.
3. Sodium thiosulphate. 99.2 grams. of the purest thiosulphate are dissolved in boiled out distilled water and the volume made up to 1 litre with boiled out distilled water. It should be prepared at least a week before it is standardised.
4. Potassium iodide. Saturated solution. 100 grams. of the solid are treated with 70 cc. of hot distilled water and the solution allowed to cool.
5. Soluble starch. Shake 1 gram. of soluble starch (see p. 301) with about 10 cc. of distilled water and pour the suspension into 90 cc. of boiling water.

**Standardisation of the thiosulphate.** Measure 20 cc. of the copper sulphate into a 200 cc. Erlenmeyer flask. Add 40 cc. of distilled water and 20 cc. of strong (33 per cent.) acetic acid. Insert a thermometer and cool or warm to  $20^\circ\text{C}$ . Run in about 6.5 cc. of the saturated potassium iodide, the thermometer being withdrawn and its stem washed with this solution. The iodine liberated is titrated at once with the thiosulphate. When approaching the end point add about 1 cc. of the soluble starch. The colour changes to a chocolate brown when very near the end point. This is best determined by the "spot test" method. Allow a drop of the thiosulphate to fall on the quiet surface of the liquid. If the end point has not been reached, a very perceptible white area is seen around the drop. This is very readily distinguished from the diminution of the slightly yellowish colour of the suspended cuprous iodide. The volume of the drop delivered by the burette must be deducted from the total volume added.

The copper value of the thiosulphate is calculated as shewn in the following example:—

20 cc. of the copper sulphate = 352.93 mg. Cu.

This required 27.6 cc. of thiosulphate.

So 1 cc. of thiosulphate =  $\frac{352.93}{27.6} = 12.78$  mg. Cu.

**The heating apparatus.** Use the apparatus shewn in fig. 15. In a 200 cc. Erlenmeyer flask of Resistance glass, and of about 6 cm. basal diameter, place 60 cc. of distilled water. The flask is fitted with a 2-hole rubber stopper carrying a thermometer so graduated that the stem above  $34^{\circ}\text{C}$ . is visible above the upper edge of the stopper. The lower end of the thermometer should be about 2 mm. from the bottom of the flask.

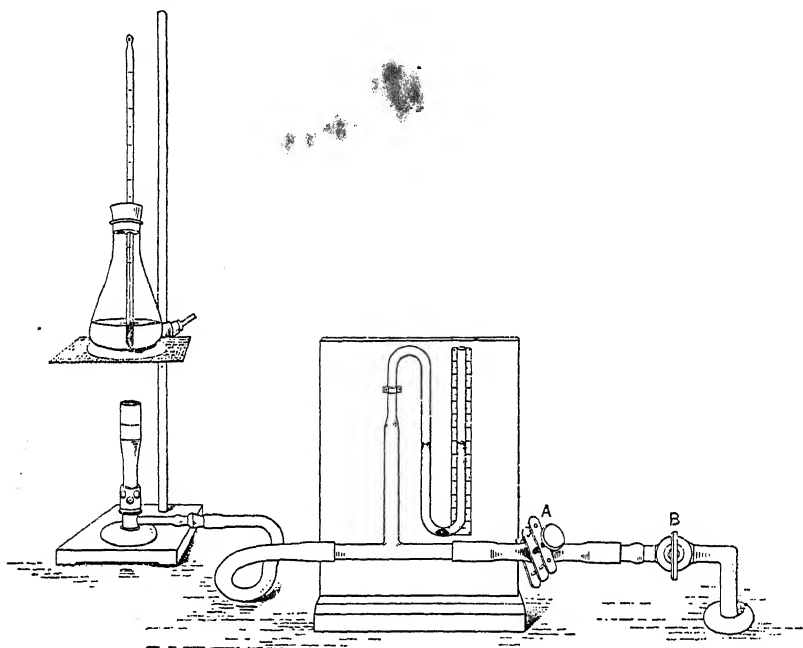


Fig. 15. Cole's apparatus for maintaining a standard heating power. The manometer tube contains a dilute solution of eosin or other dye. It also contains a globule of mercury which nearly fills the bottom of the tube. This prevents the rapid oscillations of pressure due apparently to the explosions of local gas engines.

Turn on the tap B to its full extent and light the flame of a Bunsen or Meker burner, which is placed under a piece of asbestos gauze carried by an adjustable ring stand. The gauze should be from 4 to 6 cm. above the top of the burner. Tighten the screw A till the pressure is reduced about one-third. Allow the gauze to get thoroughly heated and then place the flask in the centre of the

heated gauze. By means of a stop-watch note the time for the temperature to rise from  $35^{\circ}$  to  $95^{\circ}$ . If the time is greater or less than 120 secs. loosen or tighten the screw A and repeat the experiment with another 60 cc. of distilled water until the temperature of the water rises from  $35^{\circ}$  to  $95^{\circ}$  in  $120 \pm 2$  secs. The height of the ring and the thickness of the asbestos should be such that the pressure is well under the minimum supplied to the laboratory and yet sufficient to prevent any risk of the flame striking back. Note the manometer reading. The standard heating power can be rapidly obtained for further experiments by adjusting the screw A so that the manometer shews the requisite pressure.

**Filtrating Apparatus.** It is convenient to use the apparatus shown in Fig. 16. A is a "Duro" flask of 200 cc. capacity. Tube B

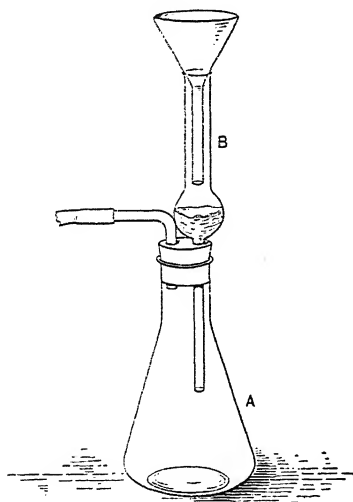


Fig. 16. Filtering apparatus for reduced copper.

is an ordinary calcium chloride tube. The lower end should reach at least 3 cm. below the lower edge of the stopper to prevent loss by splashing during filtration. The filtering mat is made of glass wool, asbestos fibre, powdered pumice and asbestos fibre added in that order. The mat should be washed with nitric acid and then thoroughly washed with water. After a test the cuprous oxide on the mat is dissolved in nitric acid diluted with an equal volume of water and then thoroughly washed.

An ordinary Gooch crucible can be used with a mat prepared in the same way. The arrangement is shewn in fig. 33, p. 259.

**Method of Analysis.** Into a 200 cc. Erlenmeyer flask measure 20 cc. of the standard copper sulphate, 20 cc. of the alkaline tartrate, and 20 cc. of the sugar solution (which must not contain

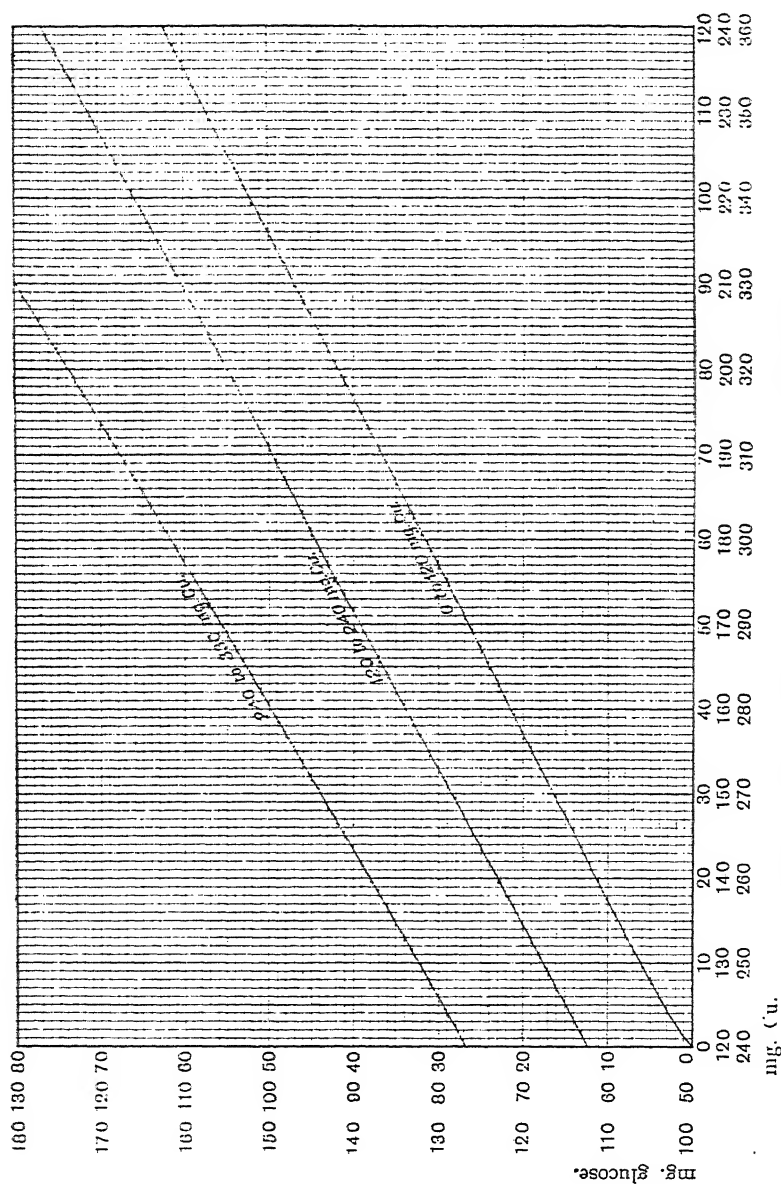


Fig. 17. Curve showing amount of copper reduced by glucose.

more than 180 mg. of glucose). Fit the two-holed rubber stopper firmly into the neck of the flask, adjust the thermometer so that its lower end is 2 mm. from the bottom of the flask and place on the heated gauze. Note the time when the mercury indicates a temperature of  $95^{\circ}\text{C}$ . Allow the heating to continue for exactly 20 secs. beyond this. Remove the flask by gripping the rubber stopper and swirl it for a second or two under the tap or in a bowl of water. The reduction of the temperature practically stops the reduction. Filter the hot fluid at once, using the stem of the thermometer as a stirring rod. Wash the flask twice with about 7 cc. of distilled water. Cool the filtrate by holding the flask under the tap. Add exactly 4 cc. of strong sulphuric acid, insert a thermometer and cool to  $20^{\circ}$ . Add 6.5 to 7 cc. of the saturated solution of potassium iodide, washing the stem of the thermometer with this solution. Titrate at once with the standardised solution of sodium thiosulphate as described above, using soluble starch as an indicator when near the end point.

**Calculation of results.** From the amount of thiosulphate required the amount of copper in the filtrate is determined. Knowing the amount taken (352.9 mg.), the amount reduced by the sugar can be calculated. The amount of glucose corresponding to this copper can be determined by a reference to the curve in Fig. 17.

**Example.** The copper in the filtrate required 14.62 cc. of thiosulphate.

1 cc. of thiosulphate = 12.86 mg. Cu.

So copper in filtrate =  $14.62 \times 12.86 = 188.0$  mg. Cu.

So copper reduced by glucose in 20 cc. =  $352.9 - 188.0 = 164.9$  mg.

From the curve this is seen to correspond to 86.3 mg. glucose.

So 20 cc. contain 86.3 mg. glucose.

So 100 cc. contain 431.5 mg. glucose. = 0.431 per cent.

**NOTE.**—If the amount of reduced copper is between 60 and 200 mg., the amount of glucose corresponding to this can be obtained by multiplying by 0.522.

#### 161A. The estimation of lactose by the copper-iodide method.

The method is exactly similar to that described in the previous exercise. The author is responsible for the

copper values for lactose. They are represented graphically in fig. 18.

It must be noted that the results are given as anhydrous lactose, and not as the crystalline hydrate.

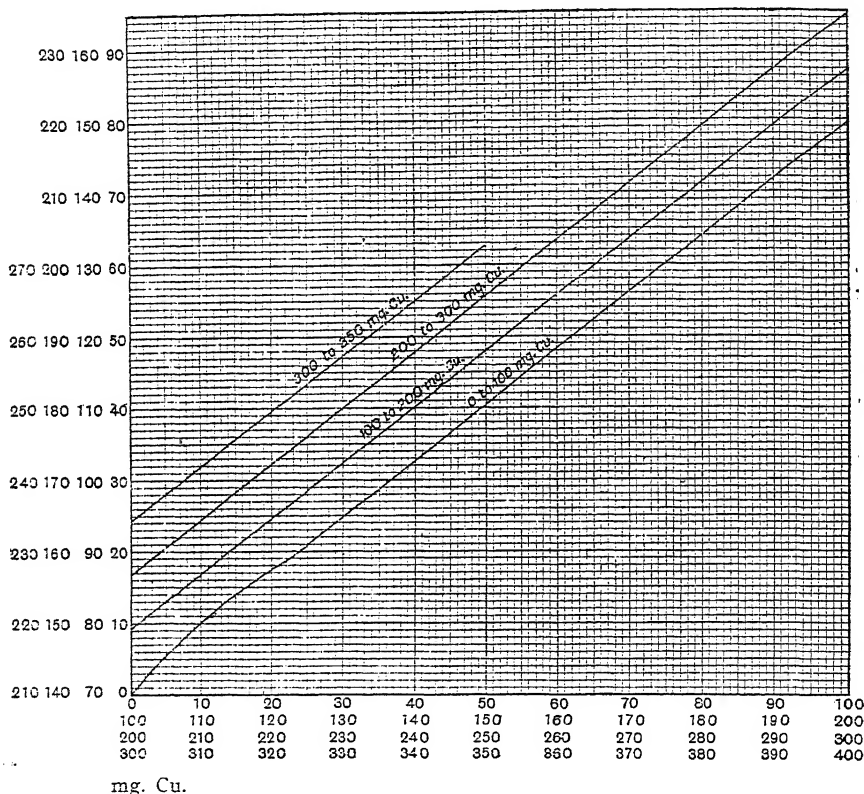


Fig. 18. Curve showing amount of copper reduced by lactose anhydride.

In the case of lactose as much as 250 mg. may be present in the 20 cc. taken.

The copper values above 25 mg. Cu. can be converted to anhydrous lactose by the use of the following formula :

$$\text{mg. anhydrous lactose} = 1.25 + \text{mg. Cu.} \times 0.778.$$



**162. Fehling's method.**

*Preparation of solution.* See Ex. 97, p. 106.

*Method.* With a pipette measure 10 cc. of freshly prepared Fehling's solution into a small flask. Add 40 cc. of distilled water, heat the mixture till it boils and *keep it boiling the whole time*. Run in the sugar solution from a burette, 0.5 to 1 cc. at a time, allowing the mixture to boil for about 15 secs. between each addition. A red precipitate of cuprous oxide forms and the intensity of the blue in the supernatant fluid decreases. Continue to add the sugar till this is completely removed. This is best determined by holding the flask by the rim at the neck and viewing it by transmitted light. If an excess of sugar be added a yellow or brown colour appears due to the formation of caramel by the action of the alkali on the sugar.

If less than 5 cc. of the sugar are used, the solution must be diluted till about 10 cc. are necessary. Thus if 2.5 cc. are used in the first rough titration, the sugar should be diluted 1 in 4, by taking 25 cc. and adding water till the volume of the solution is 100 cc. The burette is washed out and filled with this diluted solution and the process repeated. But this time run in nearly the whole of the sugar solution judged necessary at such a rate that the mixture does not go off the boil. Then add 0.1 to 0.2 cc. at a time till the reduction is complete. This titration should be repeated at least once more.

*Calculation.* 10 cc. of Fehling's solution are reduced by 0.05 gram. glucose.

*Example.* 1.5 cc. of the original solution necessary.

Sugar diluted 1 in 7 (10 cc. sugar made up to 70 cc.)

10.2 diluted sugar solution required for 10 cc. Fehling's.

10.2 cc. dil. sugar = 0.05 gm. glucose.

100 cc. " " =  $\frac{0.05 \times 100}{10.2}$  "

100 cc. original sugar =  $\frac{0.05 \times 100 \times 7}{10.2}$   
= 3.43 per cent.

**162A. Ling's method.**

*Preparation of the indicator.* Dissolve 1.5 gram. ammonium thiocyanate and 1 gram. ferrous ammonium sulphate in 10 cc. water at about 45° C. and cool at once. Add 2.5 cc. of concentrated

hydrochloric acid. The solution thus obtained has invariably a brownish-red colour, due to the presence of some ferric salt. Add zinc dust, in small portions at a time, till the fluid is just colourless. On standing for some time the red colour reappears, and must be removed again by a trace of zinc dust. But the delicacy of the indicator is impaired by being decolourised several times. When this indicator is treated with a cupric salt, the colourless ferrous thiocyanate is oxidised to the red ferric thiocyanate.

*Method of analysis.* 10 cc. of Fehling's solution and about 30 cc. of water are boiled in a flask and the sugar solution is run in from a burette as described above in Fehling's method. *The indicator is not used till the blue colour has nearly disappeared.*

Then place a drop of the indicator on a white slab. Transfer a drop of the mixture from the flask to the middle of the drop of the indicator as rapidly as possible by means of a glass tube. If a red colour appears immediately on touching the drop the reduction is not completed. More sugar must be added and a fresh drop of the indicator used as before till no colour or only a faint tinge of red is obtained. If less than 5 cc. of the sugar solution are necessary to complete the reaction, the solution must be diluted till about 10 cc. are required, as described above in Fehling's method.

*Special precautions.* Use a glass tube, not a rod, for transferring the drop.

Do not put your finger on the top of the tube. Dip it in the flask and transfer it immediately to the indicator. The flask may be taken off the boil for an instant while this is done.

Do not stir the drops on the slab.

Wash the tube before using it again.

*Calculation of results.* This is the same as in Fehling's method.

### 163. The estimation of cane sugar by Benedict's method.

Measure 50 cc. of the solution with a pipette into a flask. Add 10 cc. of N. hydrochloric acid. Boil over a free flame and keep the mixture very gently boiling for three minutes. Cool under the tap, neutralise by the addition of 10 cc. of N. sodium hydroxide. Transfer quantitatively to a 100 cc. volumetric flask and make up-

the volume to the mark with cold distilled water, rinsing the boiling flask out with small amounts of water. Mix carefully, and estimate the invert sugar by Benedict's method.

*Calculation of results.* 25 cc. of Benedict's solution = 0.0475 gram. hydrolysed cane sugar. The concentration found must be multiplied by 2, owing to the dilution made in preparing the hydrolysed solution.

### E. The theory and use of the Polarimeter.

Waves of ordinary light vibrate simultaneously in all directions perpendicular to its direction of propagation. By means of certain contrivances it is possible to affect the light so that the vibrations proceed in a single plane. Such light is *plane polarized*. The plane in which the light waves vibrate is called the *plane of polarization*. This conversion of ordinary light into polarized light is generally brought about by means of a modified prism of Iceland spar known as a Nicol's prism. If a beam of light (fig. 19) falls on the

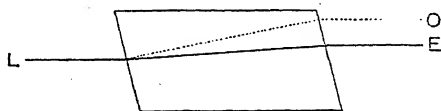


Fig. 19. Crystal of Calc Spar.

face of a rhombohedron of Iceland spar it divides on entering into two rays, unequally bent, both of which are polarized, their planes of polarization being at right angles to one another.

The extraordinary ray (E) is the lesser refracted ray: the ordinary ray (O) is the more refracted ray. Before the calc spar can be utilised for polariscopic purposes one of the rays must be eliminated. This is best effected by Nicol's method of splitting down a crystal in a certain plane, grinding down the natural ends to reduce the acute angles from  $71^{\circ}$  to  $68^{\circ}$ , and uniting the faces by Canada balsam (fig. 20).

A beam of light entering parallel to the long sides of the prism is resolved into its two component rays. The more refracted (ordinary) ray (O) meets the film of Canada

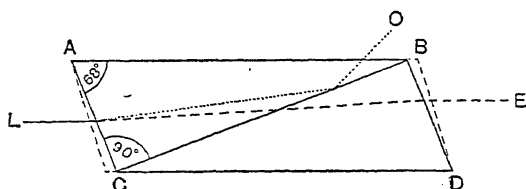


Fig. 20. Diagram of refraction in a Nicol's prism.

balsam (CB), and is completely reflected and absorbed by the black varnish usually placed on the sides of the prism. The other component (the extraordinary ray) (E) passes through the film of balsam and emerges in a polarized condition from the end surface of the Nicol.

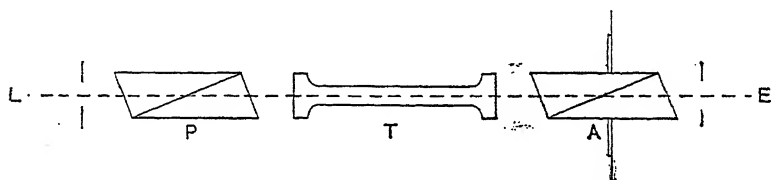


Fig. 21. Plan of arrangement of a simple polarimeter.

In a polarimeter (fig. 21) a second Nicol prism called an analyser (A) is used in addition. This is mounted in such a way that it can be rotated around its long axis. The polarized ray that emerges from the polarising Nicol (P) falls on the face of the analyser, and will only pass through unimpeded provided that it can contrive to vibrate in the same plane. In this position the Nicols are said to be parallel. If the analyser be rotated through an angle of  $45^\circ$  the ray is completely absorbed and the Nicols are said to be crossed. On rotating through a further angle of  $45^\circ$  the Nicols are again parallel. Suppose a tube of water be interposed between the two Nicols (fig. 21) and a source of light at L be viewed through the system, the Nicols are crossed when the analyser is rotated so that the minimum

illumination is obtained. If now, instead of water, the tube be filled with a solution of glucose or of certain other substances, it will be found that the illumination is not minimal. To attain this result the analyser must be rotated through a certain angle. The reason for this is that in passing through the glucose solution the plane

of polarisation has been gradually rotated so that on emerging and striking the analyser some of the light can get through. To get the minimum illumination the analyser has to be rotated to the right through an angle equal to that through which the sugar solution has rotated the plane of polarisation of the ray that entered it.

The simple arrangement described is not sufficiently sensitive.

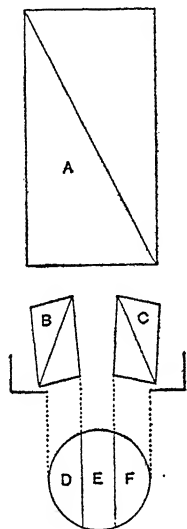


Fig. 22. Plan of a three-field polarimeter. *A* is the polarising Nicol; *B* and *C* are the small accessory Nicols that resolve the field into three parts, *D*, *E*, and *F*.

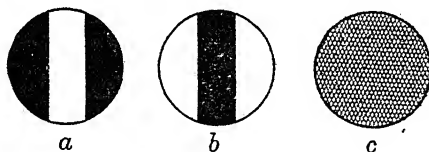


Fig. 23. The appearances seen in *a* and *b* indicate that the analyser is not in the correct position. When the analyser is correctly adjusted the three parts of the field have an equal feeble illumination as shewn in *c*.

Modern instruments have two small Nicol's prisms placed between the polariser and the solution (see fig. 22), the effect being to divide the field into three vertical sections. The zero and end points are obtained when the three fields have an equal *feeble* illumination (fig. 23, *c*). The source of illumination must be monochromatic, since the angle of rotation varies with the wave length employed. That generally used is sodium light,

obtained by heating sodium chloride or bromide in a platinum ring. The light emitted has a wave length corresponding to the D line of the solar spectrum. A much more brilliant illumination can be obtained by use of the green rays emitted from a mercury lamp. The rotation being greater with the shorter wave length, greater accuracy can be obtained.\*

The rotation varies for different substances. It is increased by increasing the concentration of the solution or the length of the tube. It also varies with the temperature, nature of the solvent, and the wave-length of the light used.

The *specific rotatory power* is the rotation observed through a tube 1 decimetre in length of a solution calculated to be 100 per cent. This is generally expressed as  $[\alpha]$ . If the sodium light is employed it is expressed as  $[\alpha]_D$ .

$$[\alpha]_D = \frac{r \times 100}{c \times l}.$$

where  $r$  = the observed rotation.

$c$  = the concentration in grams. per 100 cc.

$l$  = the length of the tube in decimetres.

If the temperature is defined, it is usually expressed by

$$[\alpha]_D 20^\circ.$$

If  $[\alpha]_D$  be known, the concentration in grammes per 100 cc. is given by

$$C = \frac{r \times 100}{[\alpha]_D \times l}.$$

The specific rotatory powers of the more common sugars is shewn below. I am indebted to Dr. Lowry for

---

\* The apparatus in the author's laboratory consists of a triple field instrument by Hilger, of London, fitted with a horizontal slit and a direct vision spectroscop. A mercury lamp is used as the source of illumination. An accuracy of  $0.01^\circ$  is easily obtained.

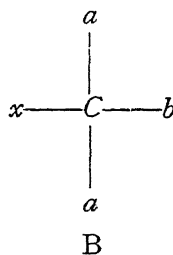
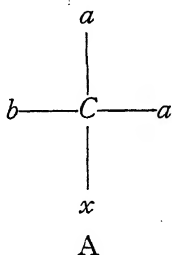
the information concerning the rotations of the sugars to the mercury green. In all cases the final rotations are given (see p. 103).

|                           |    |   |      | $[a]_D$ | $[a]_{Hg}$ |
|---------------------------|----|---|------|---------|------------|
| Glucose .. ..             | .. | + | 52.5 | 62      |            |
| Lactose hydrate ..        | .. | + | 52.4 | 61.9    |            |
| Lactose anhydride ..      | .. | + | 55.2 | 65.2    |            |
| Maltose .. ..             | .. | + | 138  | 163     |            |
| Sucrose .. ..             | .. | + | 66.5 | 78.5    |            |
| Fructose .. ..            | .. | - | 93.8 | - 110.8 |            |
| <i>d</i> -Galactose .. .. | .. | + | 81   | 95.7    |            |
| <i>l</i> -Xylose.. ..     | .. | + | 19   | 22.4    |            |
| Invert sugar .. ..        | .. | - | 20.6 | - 24.6  |            |

For carbohydrates the  $[\alpha]_{Hg}$  can be obtained by multiplying  $[\alpha]_D$  by 1.181. This relationship is not necessarily true for substances whose molecular construction differs from that of the carbohydrates.

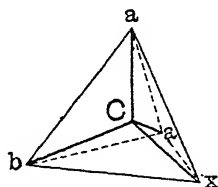
#### F. Optical Activity and the Asymmetric Carbon Atom.

If C be a carbon atom attached to *a*, *b* and *x*, different atoms or groups, it is found that there exists only one modification of the type  $Ca_2bx$ . But if the structural formula be written in one plane it would appear that two arrangements are possible, viz.



In A the groups *a* are adjacent, whilst in B they apparently are separated.

The accepted explanation of the facts is that the carbon atom possesses four valencies or bonds directed towards the apices of a tetrahedron, the carbon atom being at the centre.



The student is advised to construct such a model from a piece of plasticine and four matches, the central piece of plasticine representing the carbon atom and the matches the four bonds attached to it. The heads of the matches should be left plain or marked with little balls of variously coloured plasticine to indicate the different groups attached to the central carbon atom. Such a model is represented in fig. 24.

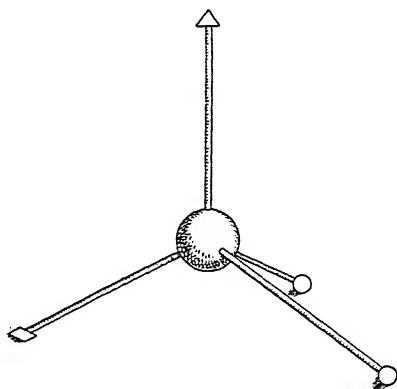


Fig. 24. Model of carbon atom attached to 3 different groups, representing the compound  $Ca_2bx$ .

Another identical model should now be prepared. It will be found that the two can be superposed, as shewn in fig. 25.

Now change the positions of any two matches in one of the models. It will be found that the two models can



still be superposed. In fact, no matter how the matches

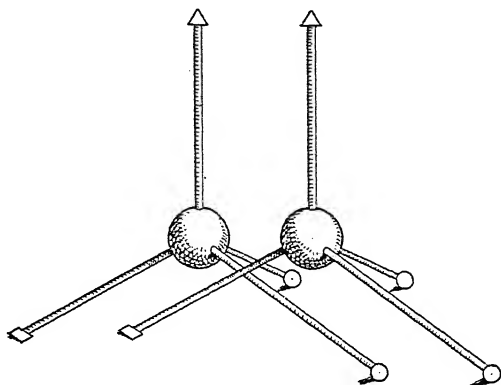


Fig. 25. Two identical superposable models of the type  $Ca_2bx$ .

are changed about only one arrangement is possible. This

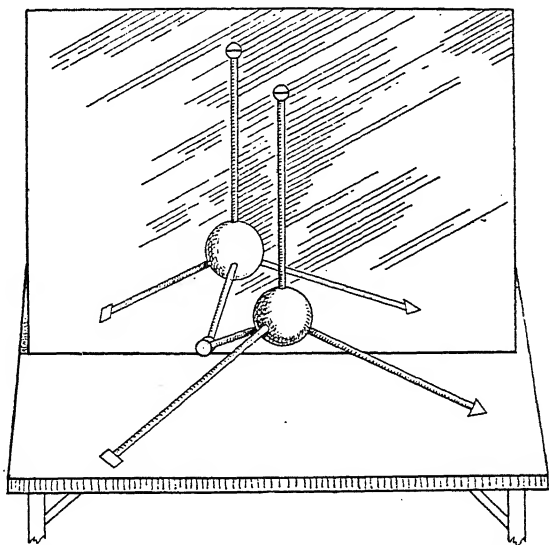


Fig. 26. Model of an asymmetric carbon atom and its mirror image.

is in agreement with the fact that only one modification of the type  $Ca_2bx$  exists.

Now make two exactly similar models in each of which the carbon atom is represented as being united to four different groups. Being exactly similar the two models are naturally superposable. Now change the position of any two matches in one model only. The two models thus formed cannot be superposed. On examination it will be found that the two models have a relationship to one another similar to that of the right to the left hand, or of one object to its image in a mirror. This is represented in fig. 26.

If now still another model be constructed it will be found that it can be superposed on one or other of the two previous ones. That is, there exist two modifications and two only, of compounds of the type  $Cabxy$ .

If the model of the type  $Ca_2bx$  be examined it will be seen that it can be divided into two symmetrical halves. The plane of symmetry and method of division is indicated in fig. 27. It must be understood that though a plane of symmetry exists the atoms or groups are not actually split into halves by it. An examination of the figures shewn

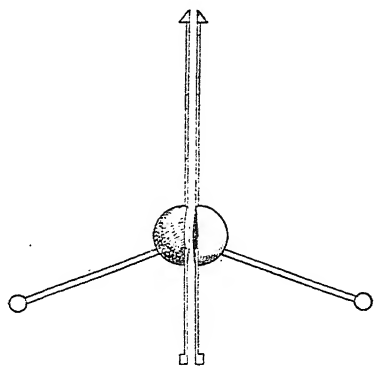


Fig. 27. Plane of symmetry of model shewn in Fig. 24.

in fig. 26 will reveal the fact that they do not possess a plane of symmetry. It has been ascertained that all compounds of the type  $Cabxy$  exist in two modifications. The solutions of one of these rotates the plane of polarisation to the right: that of the other exactly the same degree to the left. The former is the "*dextro-rotatory*" or the *d*-compound: the latter is the "*laevo-rotatory*" or *l*-compound.

These are sometimes known as *enantiomorphs*. Since the compound has no plane of symmetry a carbon

atom attached to four different groups is known as an *asymmetric carbon atom*. The possession of an asymmetric carbon atom in a compound is essential to the possession of optical activity by that compound.

If equal parts of the *d*- and *l*- varieties of a compound be mixed together, the solution of the substance is "optically inactive by external compensation." Such an inactive mixture is known as "racemic," and is designated by *dl*- or *i*-. When a compound that contains an asymmetric carbon atom is synthesised, it is always found that equal parts of the *d*- and *l*- varieties are formed. These can often be *resolved* into their active constituents by various methods, the most interesting of which is the *biochemical*, depending on the property of living organisms of *selective assimilation*, one of the two components being destroyed more rapidly than the other. A few examples of this are given below.

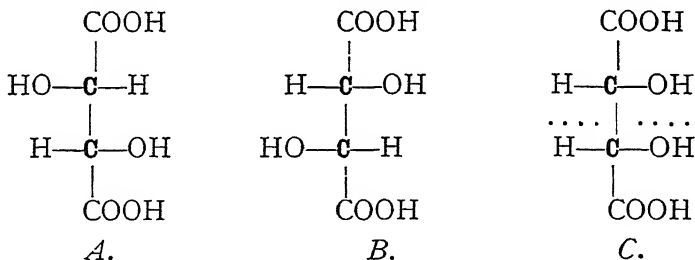
| Substance                                | Organism         | Destroyed  |
|--|------------------|------------|
| Lactic acid                              | penicillium      | <i>d</i> . |
|  | bacteria         | <i>l</i> . |
| Glyceric acid                            | penicillium      | <i>l</i> . |
|  | bac. ethaceticus | <i>l</i> . |
| Amyl alcohol                             | fungus           | <i>l</i> . |
| Glucose, mannose,<br>galactose, fructose | yeast            | <i>d</i> . |
| Racemic acid                             | penicillium      | <i>d</i> . |
|  | schizomycetes    | <i>l</i> . |
| Leucine                                  | yeast            | <i>l</i> . |
| Alanine                                  | yeast            | <i>d</i> . |

This power of selective assimilation finds a parallel in the different physiological action of enantiomorphs on the body, and of the body and also of enzymes on enantiomorphs. For instance, *dl*-adrenaline has only slightly more than one-half the physiological activity of the natural

*l*-adrenaline: *d*-asparagine has a sweet taste, whilst *l*-asparagine is insipid: *l*-nicotine is far more poisonous than *d*-nicotine. Further, if *dl*-phenyl-aminoacetic acid be administered to an animal, only the *l*- variety is found in the urine, the body having the power to destroy the *d*-acid. Many other similar instances have been described.

Proteins, like casein, can be racemized by treatment with dilute alkalis. Such proteins are not attacked by the proteolytic enzymes, and when administered to dogs can be recovered quantitatively from the fæces. The relationship between configuration and enzyme action is discussed on p. 184.

In a few cases substances having two, four or six asymmetric carbon atoms are optically inactive, and cannot be resolved into two components. The optical inactivity is due to *internal compensation*, the molecule possessing a plane of symmetry. The simplest example of this is that of mesotartaric acid. We can represent the formulæ of the tartaric acids in one plane as follows:—



*d*-Tartaric Acid.    *l*-Tartaric Acid.    Mesotartaric Acid.

The dotted line in the structural formula of Mesotartaric Acid indicates the plane of symmetry. *A* can be regarded as the mirror image of *B*. A mixture of these in equal parts will be inactive through external compensation. If the upper carbon atom of *C* be regarded as dextro-rotatory, then the lower one can be regarded as its mirror image and will therefore be laevorotatory. The whole molecule will therefore be optically inactive, and the compound is incapable of being resolved into two constituents.

## CHAPTER VI.

### THE FATS, OILS AND LIPINES.

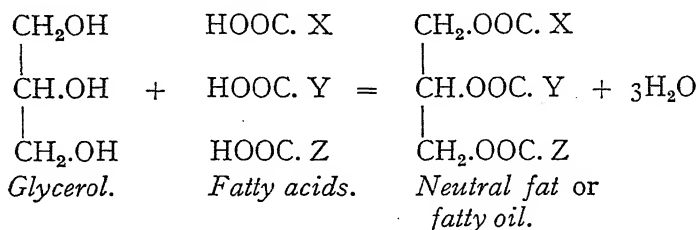
These compounds are characterised *physically* by having low melting points, a greasy feel, and by being insoluble in water; but soluble in ether, alcohol, chloroform, and certain other organic solvents, and *chemically* by being mainly composed of the radicles of the higher fatty acids. A certain number of substances with the appropriate physical properties have been found to belong chemically to the terpenes.

The following classification (after Gies) include all those of known physiological interest:

1. **Fats.** Neutral glycerides of fatty acids, solid at 20°C.
2. **Fatty oils.** Neutral glycerides of fatty acids, liquid at 20°C.
3. **Essential oils.** Volatile substances of varied chemical nature, *e.g.* oil of cloves.
4. **Sterols.** Alcohols of the terpene group, *e.g.* cholesterol.
5. **Waxes.** Esters of sterols and fatty acids, *e.g.* beeswax and spermaceti.
6. **Phospholipins** or phosphatides. Compounds of fatty acids containing phosphorus and nitrogen, *e.g.* lecithin, kephalin.
7. **Galactolipins** or cerebrosides. Compounds of fatty acids, galactose and a nitrogenous complex.

The *fats and fatty oils* are glycerol esters of the higher fatty acids.

An ester is a compound formed by the condensation of an alcohol with an acid. Glycerol, being a trivalent alcohol, can condense with three molecules of a fatty acid.



The three radicles, X, Y and Z may be the same, or they may differ.

The *fatty acids* most commonly found in the composition of these substances are

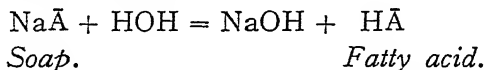
Palmitic acid,  $\text{C}_{15}\text{H}_{31}\text{.COOH}$   
or  $\text{CH}_3\text{.(CH}_2\text{)}_{14}\text{.COOH.}$

Stearic acid,  $\text{C}_{17}\text{H}_{35}\text{.COOH}$   
or  $\text{CH}_3\text{(CH}_2\text{)}_{16}\text{.COOH.}$

Oleic acid,  $\text{C}_{17}\text{H}_{33}\text{.COOH}$   
or  $\text{CH}_3\text{(CH}_2\text{)}_7\text{.CH = CH.(CH}_2\text{)}_7\text{.COOH.}$

As can be seen from the formulæ, the first two are saturated acids of the acetic acid series, whilst oleic acid is unsaturated and belongs to the acrylic acid series. Occasionally other acids, more unsaturated than oleic acid, are found, such as linoleic acid.

Palmitic acid melts at  $62\cdot6^\circ\text{C.}$ ; stearic acid at  $69\cdot3^\circ\text{C.}$ ; oleic at  $14^\circ\text{C.}$ , solidifying at  $4^\circ\text{C.}$  They are all insoluble in water, and only slightly soluble in cold alcohol, with the exception of oleic acid, which dissolves readily. They are all freely soluble in ether. The sodium and potassium salts of these acids are known as *soaps*, which are readily soluble in water. If these salts be diluted with water they are hydrolytically dissociated.



Since the fatty acid is only slightly ionised, and is insoluble in water, dilution of a clear solution of sodium stearate causes the liberation of sodium hydroxide and of the fatty acid, the latter causing the solution to become opalescent. This reaction is not so well marked with the oleates. This hydrolytic dissociation is checked by the addition of alcohol. For this reason it is essential to have alcohol present to the extent of 50 per cent. at the end of a titration of fatty acids with aqueous alkalis.

The calcium, magnesium, barium and lead salts or soaps are insoluble in water.

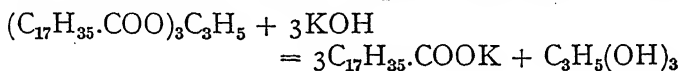
The *glycerides* formed by these three acids are known as tripalmitin, tristearin and triolein respectively. The melting points are  $66^{\circ}$ ,  $71^{\circ}$  and  $-5^{\circ}\text{C}$ . In the body they are found mixed in different proportions, and possibly some of the compounds have more than one fatty acid in the molecule. They are hydrolysed by boiling acids and alkalis, by superheated steam, and by certain enzymes called lipases or steapsins. If an alkali be used as the hydrolytic reagent, the fatty acid liberated combines with the alkali to form a soap. This special form of hydrolysis is therefore called *saponification*. The most rapid method of effecting this hydrolysis in the laboratory is by boiling the fat with an alcoholic solution of soda or potash. The fat being soluble in the alcohol the formation of ethyl esters of the fatty acids proceeds rapidly. The ethyl esters are themselves hydrolysed by the water present, and so the fat is completely converted into glycerol and soap.

Various methods have been devised for the identification of the fats and oils, amongst them being:

1. The *melting point*, or the solidifying point.
2. The *saponification value*. A known weight of the fat is heated with a given volume of a standardised alcoholic solution of potash. The mixture is then titrated with standard hydrochloric acid against phenol phthalein, alcohol being added to maintain a concentration of 50 per cent. The number of milligrammes of potassium hydroxide

that have been neutralised by the free or combined fatty acids of 1 gram. of the fat is the value required.

The saponification value of pure tristearin is 189.



Mol. wt. 890

$3 \times 56$

1 gram. requires 0.189 gram. KOH.

The value for triolein is 190, and for tripalmitin 208.

The saponification value is a measure of the mean molecular weight of the fatty acids constituting the fat. It is increased by a decrease in the molecular weight. It is lowered by the presence of unsaponifiable substances, such as cholesterol.

3. The *iodine value*. Oleic acid is an unsaturated acid, and can combine with two atoms of iodine. The saturated acids and their glycerides do not absorb iodine. The iodine value is the grams. of iodine absorbed by 100 grams. of the fatty material. Thus, triolein has a molecular weight of 884, and can absorb 6 atoms of iodine per molecule. So that 884 grams. absorb  $6 \times 127 = 762$  grams. of iodine, or 86.2 per cent. Since some fats contain radicles with more than one double bond, it is clear that the iodine value will not determine absolutely the character of a fat.

### *The emulsification of the fats.*

Fats can be emulsified, *i.e.* broken up into droplets, either mechanically by agitation, or "spontaneously."

"Spontaneous" emulsification takes place when a melted oil or fat that contains a certain percentage of free fatty acid is brought into contact with an alkali. The fatty acid dissolves in the alkali to form a soluble soap, and the diffusion currents thus set up break the globule of fat into small particles, the process being maintained by the continual exposure of fatty acid to the alkali. The



fat in the small intestine is thus emulsified as a preliminary to complete hydrolysis by the pancreatic lipase.

*The digestion of fats.*

The fats are hydrolysed to a small extent in the stomach by gastric lipase. This action is greater if the fat be given in an emulsified form, as in milk.

In the duodenum, the fat mixed with the fatty acid is spontaneously emulsified by the alkaline bile, succus entericus and pancreatic juice. The emulsified fat is then completely hydrolysed to glycerol and fatty acids by the pancreatic lipase. The fatty acids are converted into soluble soaps by the alkalies present. The soaps and glycerol are absorbed into the epithelial cells bordering the villi, where they are resynthesised into fats. These are passed into the lacteals, and reach the blood stream by way of the thoracic duct.

164. (a) Carefully allow a drop of neutral olive oil to fall *gently* on to the surface of some 0.25 per cent. sodium carbonate contained in a watch-glass. The drop of oil remains quite clear, and forms a thin (circular) film on the surface.

(b) Shake 4 cc. of neutral oil with 3 drops (only) of oleic acid in a *dry* test-tube. With a drop of this mixture repeat (a), using a fresh watch-glass full of  $\text{Na}_2\text{CO}_3$ . The rancid oil slowly spreads out in an amoeboid fashion and becomes converted into a milky emulsion.

(c) To the remainder of the mixture of oil and oleic acid add 12 more drops of oleic acid, shake well, and repeat the experiment. The drop becomes white and opaque, but does not become emulsified.

NOTES.—1. It is absolutely essential that the oil be quite neutral, and this can best be tested by dropping it on to 0.25 per cent.  $\text{Na}_2\text{CO}_3$ . If a spontaneous emulsion is formed, a fresh sample must be obtained, or melted fresh butter substituted.

2. The spontaneous emulsion in (b) is caused by the trace of oleic acid dissolving in the alkali to form a soap, diffusion currents being thus set up which divide the fat into microscopic droplets.

3. It is important to mix the oil very thoroughly with the oleic acid.

4. In (c) the large excess of oleic acid leads to the opaque ring of soap being formed round the oil, and this soap, being only slightly soluble in water, prevents the formation of an emulsion.

165. Shake a few drops of olive oil with 5 cc. of ether in a dry tube. The oil completely dissolves. Repeat the experiment with alcohol instead of ether. The oil dissolves partially, but is not so soluble in alcohol as in ether. Pour the alcoholic solution into water. The fat is precipitated as an emulsion.

166. Touch a piece of writing paper with a glass rod that has been dipped in olive oil. The paper is rendered translucent.

**Preparation of pancreatic lipase.** A perfectly fresh pig's pancreas is freed from fat, weighed, finely minced and ground with sand. It is then treated with three times its weight of water and its own weight of strong alcohol. It is allowed to stand for three days at room temperature and strained through muslin. It must not be filtered. When not in use it should be kept in a refrigerator. It will remain active for a considerable time.

**NOTE.**—Pancreatic lipase is a ferment that only acts with the co-operation of a co-ferment, which is soluble in water and not destroyed by boiling. Bile salts and certain other substances can act as the co-ferment. The ferment proper is practically insoluble in water, and is destroyed by boiling. If the pancreatic extract be filtered, neither the precipitate nor the filtrate has any appreciable action on fats; but when the two are mixed the original lipolytic action is recovered. The precipitate is the ferment; the filtrate contains the co-ferment.

**Preparation of an Emulsion of Fat.**—Commercial olive oil (which contains some free oleic acid) is treated in a flask with 1 drop of a 1 per cent. alcoholic solution of phenolphthalein for every 10 cc. of oil. Decinormal sodium hydroxide is added, with frequent shaking, till the mixture is slightly alkaline, as shewn by a very faint pink tinge. A very stable emulsion is thus formed, and thus a considerable surface of fat is exposed to the action of the ferment.

### Fat-splitting action of lipase (steapsin).

167. Label three test-tubes A, B, and C.

To A add 2 cc. of the pancreatic extract and 1 cc. of water.

„ B „ „ „ , boil and add 1 cc. of water.

„ C „ „ „ and 1 cc. of 1 per cent. bile salts.

It is advantageous to have the tubes fitted with well-fitting rubber stoppers. To each add 5 cc. of the emulsion of oil, shake thoroughly, and place in a water bath at 40° C. for 1 hour. Shake the tubes thoroughly every fifteen minutes. At the end of the digestion transfer the contents to three labelled beakers. Add 10 cc. of 96 per cent. alcohol to the tube, shake well, and transfer the alcoholic

washings to its appropriate beaker. Repeat this with another 10 cc. of alcohol. To each beaker add 5 drops of 1 per cent. phenol phthalein and titrate with 0.1 N. NaOH to a faint definite pink. The results vary considerably with different preparations, but the following may be taken as an example:

A required 6.7 cc. of 0.1 N. NaOH

B „ 2.3 „ „

C „ 14.9 „ „

$6.7 - 2.3 = 4.4$  is a measure of the amount of fatty acid produced in A.

$14.9 - 2.3 = 12.6$  is a measure of the amount of fatty acid produced in C.

It will be found that the presence of the bile salts materially aids the digestion of the fat.

168. **Detection of lipase.** Boil about 5 cc. of milk to destroy bacilli that may ferment the lactose. Cool and add 2 cc. of the pancreatic extract. Add about 1 cc. of a 0.01 per cent. solution of phenol red (see p. 24), and then enough of a 2 per cent. solution of sodium carbonate to give the solution a distinct reddish tinge. Divide into two portions, A and B. Boil B, and then cool it under the tap. Place the tubes in a warm bath at  $40^{\circ}\text{C}$ ., and examine at intervals. A will become yellow if lipase is present, owing to the hydrolysis of the emulsified fat of the milk into fatty acids.

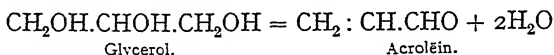
NOTE.—The hydrolysis of casein by trypsin leads to a slight increase in the concentration of hydrogen ions. For that reason it is preferable to use a mixture of cream and water instead of milk.

### Glycerol.

169. Treat a drop or two of glycerol in a test-tube with a solution of copper sulphate and then with sodium hydroxide. A blue solution is obtained, glycerol preventing the precipitation of cupric hydroxide. (See Ex. 96, note 3.)

170. Boil the solution thus obtained. Reduction does not occur

171. Heat strongly a drop or two of pure glycerol with solid potassium hydrogen sulphate in a dry test-tube. The pungent odour of acrolein (acrylic aldehyde) is noticed.



Glycerol.

Acroléin.

172. Treat about 5 cc. of a 0.5 per cent. solution of borax with sufficient of a 1 per cent. alcoholic solution of phenolphthalein to produce a well-marked red colour. Add a 20 per cent. aqueous solution of glycerol drop by drop, until the red colour is just discharged. Boil the solution: the colour returns, provided that an excess of glycerine has not been added (**Dunstan's test for glycerol**).

NOTES.—1. Any ammonium salt will discharge the colour, but in this case it does not return on heating.

2. Any polyhydric alcohol is likely to give the same reaction. The sugars are all polyhydric alcohols, but are distinguished from glycerol by their reducing properties, etc., and by the fact that they are not volatile when distilled by steam.

3. The probable explanation of the reaction is as follows: Sodium borate is partially hydrolysed in aqueous solution to boric acid and sodium hydroxide. Boric acid being a weak acid is only feebly ionised and therefore the solution reacts alkaline. On adding glycerol, glyceroboric acid is formed. This is a strong acid and hence the reaction of the solution changes from alkaline to acid. On heating, unless a large excess of glycerol be present, the glyceroboric acid is hydrolysed to glycerol, and boric acid, and the solution again becomes alkaline.

### The Higher fatty acids and their salts, the soaps.

173. Shake a few drops of oleic acid with 5 cc. of water, ether and alcohol respectively in separate tubes. The acid is insoluble in water, but soluble in alcohol or ether.

174. Place a drop of oleic acid on writing paper: a greasy stain results.

175. Shake the alcoholic solution of oleic acid with dilute bromine water. The colour of the bromine is discharged, owing to the unsaturated acid absorbing the halogen till it is saturated.

176. Repeat the experiment with an alcoholic solution of stearic acid or commercial "stearine" (a mixture of stearic and palmitic acids). The colour of the bromine persists, since these acids are members of the saturated series.

177. To about 10 drops of oleic acid add 10 cc. of boiling distilled water, and to the hot mixture add 10 per cent. NaOH drop by drop till the solution is clear. If an excess be added the excess of sodium ions causes a precipitate (see note below). A clear solution of a soap, sodium oleate, is formed. Divide this into three portions. To A add a few drops of strong HCl or  $\text{H}_2\text{SO}_4$  till the reaction is distinctly acid. Oleic acid separates out and rises to the surface of the tube.

To B add finely-powdered sodium chloride and shake. The soap is rendered insoluble and rises to the surface.

To C add some calcium chloride. A precipitate of an insoluble soap, calcium oleate, is produced.

NOTE.—B illustrates the principle of "salting out," which is used in the manufacture of soaps. The excess of sodium ions in the solution, produced by the addition of the sodium chloride, lowers the solubility of the sodium oleate, which is therefore precipitated.

178. Boil 2 cc. of olive oil with 5 cc. of a 20 per cent. alcoholic solution of sodium hydroxide in a basin over a *small* flame for five minutes or until the alcohol has all evaporated away. Add about 5 cc. of alcohol and heat again to dryness, stirring the whole time. Add about 50 cc. of distilled water and boil till dissolved. Add solid sodium chloride and stir. The soap formed is precipitated. Filter some off, dissolve in boiling water and repeat the experiments A, B, and C, described in the previous exercise.

**Cholesterol.**  $\text{C}_{27}\text{H}_{43}\text{OH}$  or  $\text{C}_{27}\text{H}_{45}\text{OH}$  is a secondary alcohol, which is very widely distributed in animal tissues. An isomer, known as phytosterol, is found in many vegetable oils. It was first discovered in gall stones, hence its name. A considerable amount is found in nervous tissues and in egg yolk. In blood serum it is present as an ester, as it is in "lanoline," the fatty matter obtained from sheep's wool. It is readily soluble in acetone, chloroform, ether and benzene. It is only slightly soluble in cold, but easily soluble in hot alcohol. It is soluble in the bile salts. It is insoluble in water, weak acids, and alkalies.

It crystallises from hot alcohol in rhombic plates, which often have a re-entering (notched) angle. From dry ether, chloroform and benzene it crystallises in needles.

It melts at  $145^{\circ}\text{C}$ . In chloroform solution it is laevorotatory,  $[\alpha] = -36.6^{\circ}$ .

Its chemical constitution is not yet determined, but it is probably a member of the terpene series.

**179. Preparation of cholesterol from sheep's brain.** Sheep's brain is minced, ground with sand, and intimately mixed with three times its weight of plaster of paris. After some hours the hard mass is ground and extracted three times with cold acetone by rubbing well in a mortar. The mixed acetone solutions are filtered and allowed to evaporate spontaneously. The cholesterol crystallises out and is recrystallised from boiling alcohol.

**180.** Mount a few crystals of cholesterol in water, examine under the microscope, and draw them. Note the rhombic plates, which are often incomplete at one corner. Irrigate the crystals with strong sulphuric acid: they turn red at the edges. Now add a drop of iodine solution: the crystals give a violet colour, changing to a green, blue, and finally a black.

**181. Salkowski's reaction for cholesterol.** Dissolve a little in a few cc. of chloroform; to the solution add an equal quantity of strong sulphuric acid and shake. The upper layer of chloroform becomes red, the layer of sulphuric acid yellow with a green fluorescence.

**182. Liebermann-Burchard reaction for cholesterol.** Dissolve a little cholesterol in 2 cc. of chloroform, contained in a perfectly dry tube. Add ten drops of acetic anhydride, then two drops of strong sulphuric acid, and shake. The solution becomes coloured a deep blue.

**Phospholipins or Phosphatides.** As mentioned on p. 153, these are compounds of fatty acids with phosphorus and nitrogen. Maclean\* classifies them as follows:—

(A) *Monaminophosphatides* ( $\text{N} : \text{P} = 1 : 1$ )

(a) Lecithin.

(b) Kephalin.

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\* *Lecithin and Allied Substances*, by H. Maclean (Longmans, Green & Co., 1918).

(B) *Diaminophosphatides* ( $N : P = 2 : 1$ )

Sphingomyelin.

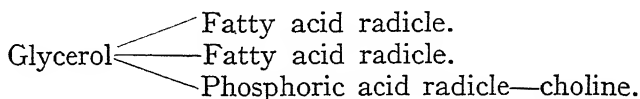
(C) *Monaminodiphosphatides* ( $N : P = 1 : 2$ )

Cuorin.

Their separation from other constituents of tissues is dependent on the fact that though they are soluble in ether they are insoluble in acetone.

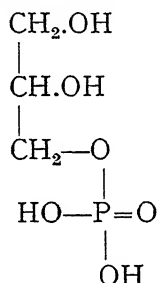
**Lecithin** is the best known member of the series. The following account of its properties is abridged from Maclean's valuable monograph. It is a yellowish-white waxy substance, which on exposure to the air absorbs oxygen and soon assumes a dark brown colour. It is very hygroscopic and in the presence of moisture forms a soft plastic mass. It dissolves very easily in alcohol, ether, chloroform, benzene, petroleum ether and many other organic reagents: also in aqueous solutions of bile salts. It is insoluble in acetone and methyl acetate. In contact with water it swells up and ultimately forms a slimy emulsion or colloidal solution, from which it is readily precipitated by salts with divalent cations, such as calcium and magnesium; salts containing monovalent cations, such as sodium chloride, act in the same way, but more slowly. In the presence of a small amount of sodium chloride, acetone readily precipitates lecithin from its emulsions with water. It is also readily precipitated from ether or chloroform solution by this reagent. On treatment with alkalis or acids it is hydrolysed, quickly on heating and more slowly in the cold. Lecithin combines with acids and bases. It also combines with certain salts of the heavy metals, such as cadmium chloride, platinum chloride, and mercuric chloride. Lecithin-cadmium-chloride is almost insoluble in alcohol, but dissolves in a mixture of carbon disulphide and ether or alcohol. It is probable that the greater part of the lecithin of tissues exists in some kind of combination with protein. Lecithin is dextro-rotatory.

The constitution of lecithin can be represented as follows :—

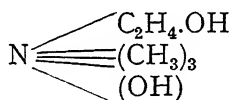


It can be regarded as a complex of glycerophosphoric acid with fatty acids and with choline.

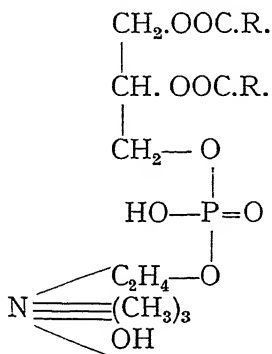
Glycerophosphoric acid is



Choline is



so that if the fatty acids be represented by  $\text{R}.\text{COOH}$ , the constitutional formula of lecithin may be





The nature of the fatty acids is not yet determined. It is possible that they differ with different specimens. They seem to be unsaturated acids of the  $C_{16}$  or  $C_{18}$  series.

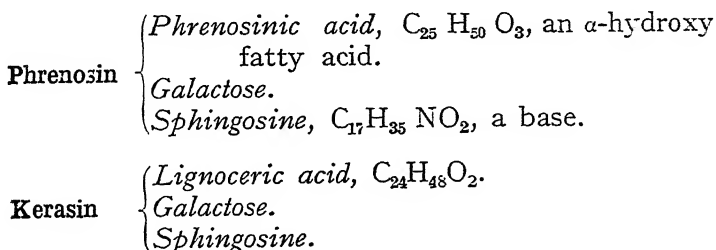
**Choline** or trimethyl- $\beta$ -hydroxy-ethyl-ammonium hydroxide is of considerable interest, since it is closely related chemically to muscarine, a very poisonous base obtained from certain fungi. In fact, pseudo-muscarine, which somewhat resembles muscarine, has recently been shewn to be the nitrous acid ester of choline. Our present knowledge concerning choline and related substances will be found in Barger's "The Simpler Natural Bases" (Longmans, Green and Co., 1914).

**Kephalin** differs from lecithin in being insoluble in alcohol. Chemically, it differs in that the base united to the phosphoric acid radicle is not choline, but oxyethylamine,  $NH_2 \cdot CH_2 \cdot CH_2OH$ .

The preparation of lecithin is described by Maclean in his monograph.

#### The Galactolipins or Cerebrosides.

These compounds do not contain phosphorus. Their name is derived from the fact that they yield galactose on hydrolysis, and are particularly abundant in the brain, though they are found elsewhere in the body. Two members of this class have been described, Phrenosin and Kerasin. Their constitution can be represented as follows :



## CHAPTER VII.

### THE CHEMISTRY OF SOME FOODS.

#### A. Milk.

The composition of milk differs considerably in different animals. The percentage composition of average samples of human and cow's milk is as follows :—

|             | Protein. | Fat. | Lactose. | Salts. |
|-------------|----------|------|----------|--------|
| Human .. .. | 1·2      | 2·7  | 6·5      | 0·2    |
| Cow's .. .. | 3·4      | 4·0  | 4·5      | 0·7    |

Other differences are that in cow's milk the proportion of casein to lactalbumin is about 6 to 1, compared with 2 to 1 in human milk.

**Casein**, the chief protein of milk, is a phospho-protein. Like all proteins, it is an ampholyte, but it differs from the majority of proteins in having marked acid characters. The iso-electric point of casein (see pages 11 and 32) is about  $P_H = 4·6$ . At this reaction it has its minimum solubility. In solutions alkaline to this it forms salts with bases; in solutions acid to this it forms salts with acids. These salts are more or less soluble in water. So it can be stated that casein is insoluble in water and dilute acids, but soluble in alkalies and strong acids. Since the reaction of milk is about  $P_H = 7$  it follows that the casein is held in solution as a salt with a base. The base is probably calcium, though it is possible that a complex with a phosphate is the condition in which the casein exists naturally in untreated milk.

Casein seems to have a molecular weight of about 8900. It is readily hydrolysed by alkalies and by proteolytic enzymes into two molecules of paracasein, which has a

molecular weight of 4450. The calcium salts of paracasein are very insoluble in solutions which have a reaction between  $P_H = 4$  and  $P_H = 7$ . It therefore follows that if casein be hydrolysed to paracasein in the presence of soluble calcium salts and the reaction be between the stated limits, then the paracasein will be precipitated as an insoluble calcium salt. This is the probable explanation of the well-known phenomenon of the clotting of milk. Casein is not coagulated on boiling. But when milk is boiled a skin forms on the surface. A similar skin forms whenever a protein solution mixed with an emulsion of a fat is heated. The skin contains protein mixed with fat. If it be removed, another skin immediately forms.

183. Examine a drop of fresh cow's milk under the microscope, using a high power. Notice the highly-refractive fat globules of varying size, the smallest exhibiting the peculiar vibration known as Brownian movement.

184. Take the **specific gravity** of milk with a lactometer. It varies between 1028 and 1034.

NOTE.—When the milk is skimmed the specific gravity rises to 1037, owing to the removal of the fat which has a low specific gravity. Dilution with water lowers the specific gravity.

185. Take the **reaction** of milk by placing drops on pieces of red and blue litmus paper and then washing off with distilled water. The blue paper is usually turned red and the red paper blue, *i.e.* the milk is amphoteric in reaction.

### Casein.

186. Take 5 cc. of milk in a test-tube and dilute with distilled water until the tube is nearly full. Add three drops of strong acetic acid and mix thoroughly. A flocculent precipitate of casein is formed, which mechanically carries the fat down with it.

187. Repeat the above experiment but add 5 cc. of the strong acetic acid. Usually no precipitate or only a slight one is obtained, the casein being soluble in the excess of acid.

188. To 20 cc. of milk in a 100 cc. measuring cylinder add 65 cc. of distilled water and 15 cc. of 1 per cent. acetic acid. Mix thoroughly and allow to stand for about 5 minutes. Mix again and filter through a pleated paper. The filtrate may have to be passed through the paper again, but can usually be obtained perfectly clear. Label the filtrate A.

189. Treat some of the precipitate obtained in the previous exercise with a little water and about 1 cc. of 2 per cent. sodium carbonate. Shake vigorously in a test-tube. A milky suspension of fat in an alkaline solution of casein is obtained.

190. To a portion of this suspension cautiously add acetic acid. The casein is reprecipitated, when the solution is definitely acid to litmus.

191. Transfer some of the precipitate obtained in Ex. 188 or in Ex. 190 to a test-tube. Add about 2 cc. of "glyoxylic reagent" (Ex. 23), and then 2 cc. of pure sulphuric acid. Mix by gentle agitation. As the casein dissolves in the hot acid a fine purple glyoxylic reaction is developed, due to the presence of tryptophane in casein.

192. Heat another portion of the precipitate with Millon's reagent (Ex. 22). The precipitate turns brick red, owing to the presence of tyrosine in casein.

193. Squeeze the remainder of the precipitate obtained in Ex. 188 between filter paper to express as much fluid as possible. Reserve a portion for Ex. 195.

Place a piece about the size of a pea in a dry test-tube and add 10 drops of pure sulphuric acid. Heat over a small flame until the mass chars. Then *cautiously* add one drop of pure nitric acid, taking care that an explosive reaction does not endanger yourself or your neighbours. Heat again over the flame until white sulphuric fumes appear in the tube. (Unless the solution is heated until it fumes in the tube the temperature will not rise sufficiently for the complete and rapid oxidation of the organic material.) If the solution again chars add another drop of nitric acid with the same

precautions. This process must be repeated until the solution can be heated till sulphuric fumes are found without the solution charring. If too much of the substance has been taken and a black pasty mass is formed, it is necessary to add a few more drops of sulphuric acid. Allow the yellow solution to cool, and then add about 5 cc. of distilled water. Add strong ammonia, drop by drop, until the reaction is just alkaline, cooling under the tap during the addition. An alkaline reaction is generally indicated by the sudden increase in the colour of the solution. Add a single drop of nitric acid to make the solution acid. Add about a half volume of ammonium molybdate solution and boil. A yellow precipitate of ammonium phospho-molybdate indicates the presence of phosphorus in the casein. This is originally present in organic combination, but has been oxidised by the above method (Neumann's) into inorganic phosphoric acid.

194. Treat 5 cc. of milk with 5 cc. of saturated ammonium sulphate solution. The casein is precipitated, entangling the fat with it. Filter, labelling the filtrate B. Treat the precipitate with water. The casein dissolves. Treat this cloudy solution with acetic acid. The casein is precipitated.

NOTE.—The casein dissolves in water because it is precipitated as a salt by ammonium sulphate.

### Fats.

195. Transfer the remainder of the precipitate obtained in Ex. 188 to a dry test-tube. Shake it vigorously with 5 cc. of ether. Pipette off the ethereal solution. Heat an evaporating basin by placing it on a boiling water bath. Turn out the flame and then add the ethereal solution to the dish. The ether evaporates rapidly and leaves a small amount of fatty residue. Wipe the dish round with a piece of writing-paper. A translucent grease spot is formed.

### 196. Estimation of fat in milk by Meig's method.

To 10 cc. of milk in a 100 cc. glass-stoppered cylinder add 20 cc. of distilled water and 20 cc. of ether. Shake for 5 minutes. Add 20 cc. of 95 to 98 per cent. alcohol and shake again for 5 minutes.

Allow to stand until the mixture has separated into two layers. Remove the upper layer by the special pipette shewn in fig. 28, collecting it in a glass evaporating basin that has been weighed. Add 5 cc. of ether in such a way as to wash down the sides of the cylinder. Remove this as before. Repeat the washing with 5 cc. of ether four more times. Evaporate the mixed ethereal solution to dryness on a water bath or a special electric heater. Place the dish in a desiccator over sulphuric acid until its weight is constant.

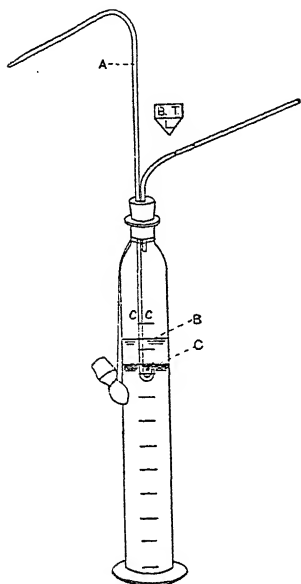


Fig. 28. Pipette arranged to remove the upper layer in Meig's method of fat extraction. The end of the tube *A* is placed just above *C*, the surface of the division between the two layers. The upper layer is then forced out through *A* by blowing air into the space above *B*.

**Important Note.**—The greatest care is necessary when evaporating off ether in open basins. It is advisable to place the dish on a boiling water bath, the flame having just previously been turned out. No other flame should be in the vicinity. When the evaporation becomes slow, remove the dish, reboil the water, turn out the flame and then replace the dish. It is safer and more convenient to use an electric heater.

### Lactalbumin.

197. Boil the filtrate *B*, obtained in Ex. 194. A coagulum of lactalbumin is obtained. (See note to Ex. 37.)

198. Examine the filtrate *A*, obtained in Ex. 188. Add a drop or two of litmus, and note that the reaction is distinctly acid. Boil, and whilst boiling add 2 per cent. sodium carbonate, drop by drop, until the reaction is only faintly acid. A coagulum of lactalbumin is formed. Filter this off and reserve the filtrate (*C*).

**NOTE.**—On boiling the acid solution, the lactalbumin is converted to metaprotein (Ex. 29). On neutralising the metaproteins are precipitated, and since the solution is boiling they are coagulated (Ex. 47). The solution must not be made alkaline, for this would cause the earthy phosphates to be precipitated.

### Lactose.

199. Boil a small portion of filtrate C with a little Fehling's solution. A well-marked reduction is obtained, due to the presence of a reducing sugar.

200. A. Measure 2 cc. of filtrate C into a test-tube. Add 3 drops of glycerol; add 10 drops of 20 per cent. copper sulphate by means of a Dreyer's dropping pipette (fig. 5), and then 2 cc. of 20 per cent. sodium hydroxide. Boil the mixture and keep it boiling for one minute. Allow the tube to stand. If the supernatant fluid is blue, repeat the experiment with less copper. If the fluid is yellow, repeat with more copper. The approximate amount of copper that is reduced by the sugar in 2 cc. of the fluid is thus found.

B. Measure 2 cc. of filtrate C into a test-tube and add 0.5 cc. of pure concentrated hydrochloric acid. Boil gently over a small flame for two minutes. Cool and add 14 drops of the copper sulphate, and 3 drops of glycerol. Neutralise by means of 20 per cent. sodium hydroxide, the neutral point being indicated by the appearance of a grey precipitate. Now add a further 2 cc. of the sodium hydroxide and boil for one minute. The whole of the copper is usually reduced. The increase in the reducing power after boiling with hydrochloric acid demonstrates that the sugar present in milk is not glucose (see Ex. 103).

201. To 5 cc. of Barfoed's solution add 1 cc. of filtrate C and repeat Ex. 101. A reduction is not obtained. This experiment, in conjunction with the previous one, indicates that the sugar present is lactose or maltose.

202. Concentrate about 25 cc. of filtrate C to about 10 cc. on the water bath. Transfer this to a test-tube. Add 1 cc. of strong acetic acid and proceed as in Ex. 109. Allow the yellow solution that is obtained to cool slowly. A yellow precipitate of *lactosazone* appears. Filter through a small paper, and suspend in about 4 cc. of water. Boil. The precipitate dissolves. Allow to cool slowly and examine the precipitate under the microscope. *Lactosazone* usually crystallises in solid ovoid clumps with a projecting fringe of fine needles. ("Hedge-hog" crystals.)

### 203. The estimation of lactose in milk by the method of Folin and Denis.\*

*Principle.* Proteins do not interfere with the method of estimation of sugar devised by Folin and McElroy (Ex. 159).

*Method.* Dilute the milk 1: 4 (25 cc. to 100 cc.) for cow's milk and 1: 5 (5 cc. to 25 cc.) for mother's milk. Fill the special burette with the diluted milk (or use a burette of the usual pattern).

Into a large tube place 5 grams. of the phosphate powder, 5 cc. of the 6 per cent. copper sulphate, shake and boil. Add 2 cc. of the strong thiocyanate and boil again. Run in about 3.4 cc. of the diluted milk and boil gently for 4 minutes. Complete the titration as described in Ex. 161.

$$\text{Calculation. } \frac{4.04 \times \text{dilution}}{\text{volume required}} = \text{anhydrous lactose per cent.}$$

NOTE.—The average amount of lactose in cow's milk is 4.5 per cent. So about 3.6 cc. of a 1 in 4 dilution of normal cow's milk is required.

### Inorganic constituents.

204. Treat the remainder of filtrate C with two or three drops of strong ammonia and boil. A slight gelatinous precipitate of calcium phosphate is produced. Filter through a small paper. Boil 4 cc. of water, to which has been added 1 cc. of strong acetic acid. Pour the hot solution on to the filter paper, and collect the filtrate in a clean tube. To the filtrate add a solution of potassium oxalate. A white precipitate of *calcium* oxalate is formed. Treat with 1 cc. of nitric acid. The precipitate dissolves. Add a few cc. of ammonium molybdate solution and boil. A yellow crystalline precipitate is slowly formed, indicating the presence of *phosphates* in milk.

### C. Cheese.

205. Shake some grated cheese in a dry test-tube with ether, and examine the ethereal solution for fat as in Ex. 195. Fats and fatty acids are present in considerable quantity.

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\* *Journal of Biological Chemistry*, xxxiii., p. 521 (1918).



206. Pound the residue from the above in a mortar with a 2 per cent. solution of sodium carbonate and filter. Acidify the filtrate with acetic acid. A precipitate of casein is formed, which is soluble in excess of acid. To the filtrate from this apply the usual protein colour reactions: they are usually all obtained owing to the presence of proteoses, peptones and various amino-acids.

#### D. Potatoes.

207. Scrape the clean surface of half a potato with a pen-knife, keeping the scrapings as fine as possible. Place the scrapings in a beaker of water, stir well, and strain through fine muslin into another beaker. Allow this to stand for a few minutes and then note the white deposit of starch. Pour off the supernatant fluid and reserve it for the next exercise. Fill the beaker containing the starch with water, stir well, and again allow the starch to settle. By repeating this process of lixiviation the starch can be obtained quite pure. Examine a little microscopically and note the characteristic form of the grains (see Ex. 132). Heat a little with water, cool, and add iodine. A deep blue colour is obtained.

208. Filter the fluid A, and test portions of the filtrate for proteins by the usual colour tests. Only small quantities of protein are found to be present, the most marked reaction being Millon's.

#### E. Flour.

White flour from the endosperm of wheat grains contains 70 to 75 per cent. of starch, about 8 per cent. of protein and about 1 per cent. of fat. The proteins are gliadin (soluble in 70 to 80 per cent. alcohol), and glutelin (soluble in alkali). When treated with water these two proteins form a sticky mass called gluten, the viscosity being due to the gliadin. Thus grains poor in gliadin, as rice and oats, do not form dough when mixed with water.

Flour only contains glucose if germination has taken place before milling.

Whole flour is obtained from the whole of the grain, except the outer husk and outer part of the bran. It is

possible that it contains something essential to growth and general nourishment. It is not quite so digestible as white flour. The bran in it stimulates the intestine and so acts as a mild laxative.

209. Mix some wheat flour with a *little* water to form a *stiff* dough. Allow this to stand for a short while, preferably at 37° C.

Wrap a piece, the size of a chestnut, in muslin, and knead it for a few minutes in a basin of water; pour the suspension into a beaker, and note the white deposit of starch grains that settles down on standing. Examine this microscopically, noting that the grains differ considerably from those of potato-starch in being smaller, circular, and with a central hilum. Make a drawing of the grains. Boil a little with water, cool, and add a drop of iodine. The deep blue starch reaction is obtained.

210. Knead the dough thoroughly under the tap until no more starch comes through the muslin. A yellowish, sticky mass, known as gluten, is left behind. Test portions of this by the usual protein colour reactions: they are all obtained, gluten being a protein.

### F. Bread.

The dough formed by adding water to flour is impervious to the digestive juices. Before it can be used it has to be aerated and the gluten rendered porous.

A pure culture of yeast is mixed with warm water, flour and salt. The dough thus formed is thoroughly kneaded, and the mass kept warm for some hours. During this time the yeast cells multiply and convert some of the starch into glucose and this into alcohol and CO<sub>2</sub>. Also the ferment of the flour called diastase becomes active and converts some of the starch into glucose. More flour is added and the process allowed to proceed for some hours longer. The gas formed causes the mass to rise. The dough is weighed out into loaves, which after being allowed to rise once more for a certain time are heated to about 232° C. for an hour and a half. The heat kills the yeast, expands the gas bubbles, and causes the outer part of the

dough to become hardened by coagulating the proteins. It also converts starch into soluble starch and dextrin, thus forming the crust. The brown appearance of this is due to the conversion of glucose into caramel.

211. Take a piece of the crumb of a stale white loaf, rub it up finely and pound with cold water in a mortar. Strain and squeeze through muslin. A white fluid is obtained containing wheat starch grains. Filter the fluid. To a portion of the filtrate add a little Fehling's solution and boil: a well-marked reduction occurs due to the presence of glucose. To another portion add iodine: a purple colour is produced, showing the presence of erythro-dextrin. If very dilute iodine be cautiously added, a blue colour is produced at first, showing that a small amount of soluble starch is present.

Boil a small amount of the residue of the bread with water in a beaker, strain through muslin and filter. Cool and test the filtrate for starch and dextrin. (Ex. 145 to 147.)

212. Repeat the above exercise, using the crust of bread instead of the crumb. Note that glucose is absent or present in traces only: dextrin and starch are present, a considerable portion of the latter existing as soluble starch and being present in the cold water extract.

### G. Meat (Muscle).

The most important constituents of living striated muscle are:—

**Proteins.** Myosinogen and Paramyosinogen.

**Pigment.** Myohaematin.

**Fat.**

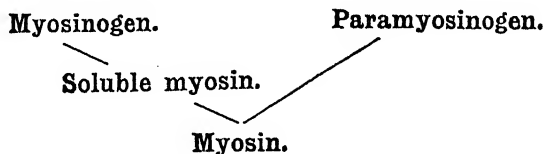
**Nitrogenous extractives.** Creatine.  
Hypoxanthine.  
Xanthine.  
Carnosine.

**Non-nitrogenous extractives.** Glycogen.  
Sarcolactic acid.

**Inorganic.** Water.  
Salts, chiefly potassium and magnesium phosphates.

The **proteins** of living muscle are mainly **myosinogen** (80 per cent.) and **paramyosinogen** (20 per cent.). The former is an albumin, coagulating at 57° C. The latter is a globulin, coagulating at 47° C.

On standing or on treatment with dilute acids they are converted into **myosin** the protein of dead muscle. In this transformation, myosinogen passes through an intermediate stage of **soluble myosin** which coagulates at 40° C.



213. **Preparation of fresh muscle extract.** A rabbit is killed, a cannula fixed into the aorta and an opening made in the right auricle. The vessels are then washed free from blood with 0.9 per cent. sodium chloride. The muscles of the limbs are removed, rapidly minced and treated with ice-cold 5 per cent. magnesium sulphate, and the mixture left in the ice chest for about 24 hours. The extract is filtered and the following tests performed with it :

214. Take the reaction to litmus. It is generally neutral.

215. Dilute a small portion with four volumes of distilled water and leave the tube in the water bath at 37° C. for some time. A clot of myosin forms, leaving muscle serum.

216. Take the reaction of the muscle serum to litmus. It is distinctly acid, due to the production of sarcolactic acid.

217. Add some acetic acid to another portion of the extract. A precipitate of myosin occurs immediately.

218. Take 5 cc. of the extract in a test-tube: place the tube in a beaker of water, supporting it by a clamp so that it does not touch the bottom of the beaker. Heat the water with a Bunsen flame and note the temperature in the tube at which distinct coagulation occurs. It is usually at about  $47^{\circ}$  C. Filter off the coagulum of paramyosinogen and heat again. Another and larger coagulum of myosinogen occurs at  $57^{\circ}$  C.

219. **Preparation of Myosin.** Fresh veal is finely minced in a machine, stirred with a large volume of water for a quarter of an hour, strained through muslin, and the washing process repeated once more. In this way certain proteins and other substances soluble in water are removed. The veal is now collected on muslin, squeezed to remove the water, ground with sand, and extracted with five times its volume of 10 per cent. ammonium chloride for several hours at room temperature. The extract is filtered through muslin, linen, and then coarse filter paper. In this way a crude, viscid solution of myosin is obtained.

220. Boil a portion of the solution. A heavy coagulum is formed. Wash the coagulum and on it perform the protein colour reactions. They are all obtained.

221. Pour 100 cc. into a litre of water contained in a tall cylinder; mix well, and note the precipitation of myosin, due to the reduction in the concentration of salts.

Allow this to settle, and then pour or pipette off as much of the supernatant fluid as possible. A suspension of myosin in dilute ammonium chloride is thus obtained for the next three experiments.

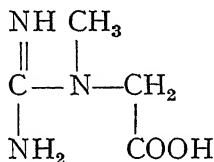
NOTE.—If this suspension be allowed to stand it slowly becomes converted into an insoluble variety.

222. To a portion add a saturated solution of common salt, drop by drop. The precipitate dissolves. Add solid NaCl to saturation: the myosin is reprecipitated.

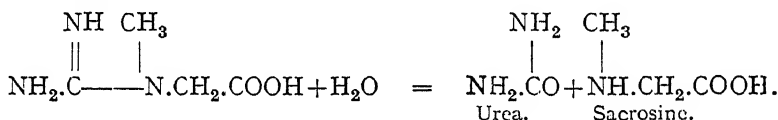
223. To a portion add saturated ammonium sulphate till the precipitate just dissolves. Now add an equal bulk of saturated ammonium sulphate. The myosin is reprecipitated.

224. Dissolve in a little ammonium sulphate and take the temperature at which the myosin coagulates. It coagulates at about 57° C. (see Ex. 218).

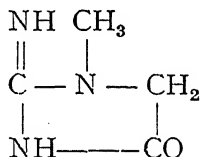
**Creatine.**—This is the most abundant nitrogenous extractive in muscle, being present to the extent of about 0.4 per cent. Chemically it is methyl-guanidine-acetic acid.



On hydrolysis with baryta water it is converted into urea and sarcosine (methyl glycine).



On being boiled with mineral acids it is dehydrated to creatinine.



Creatinine is found in normal human urine, but creatine only under abnormal conditions.

225. **Separation of creatine from meat extract.** Dissolve 10 grams. of commercial meat extract in 200 cc. of water. Add slowly a saturated solution of lead acetate till no further precipitate is formed, carefully avoiding an excess. This is best done by filtering samples and testing them with lead acetate. Filter off the precipitate of proteins and phosphates. Warm the filtrate and decompose the soluble lead compounds by means of a stream of sulphuretted hydrogen. Warm and filter off the precipitate of

lead sulphide. Evaporate the filtrate, filtering off any sulphur or sulphide that may be deposited. Continue the evaporation till a syrup is obtained. Allow this to stand in the ice chest for two or three days. Creatine separates out, mostly as oblique rhombic crystals. Examine a few under the microscope. Treat the syrup with 200 cc. of 88 per cent. alcohol, stir thoroughly with a glass rod and filter through a small paper. The creatine remains on the paper, the alcoholic filtrate containing the purine bases.

**NOTE.**—Many specimens of commercial meat extract contain creatinine as well as creatine. Rabbit's muscle is the best source of pure creatine. The muscle is finely minced, extracted with boiling water, the proteins removed by boiling and adjusting the reaction. The filtrate is worked up as described above.

**226. Conversion of creatine into creatinine.** Dissolve the creatine in about 30 cc. of hot water and divide the solution into two equal portions, A and B. Treat B with an equal volume of normal HCl and heat on a boiling water bath in a flask fitted with a cork and long glass tube (to act as an air condenser) for three to five hours. The creatine is converted into creatinine. Neutralise the solution with caustic soda.

Test A and B for creatinine by the following tests:

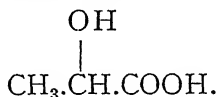
**227. Jaffe's test for creatinine.** Treat 10 cc. of the solution with 15 cc. of saturated picric acid solution and 5 cc. of 10 per cent. caustic soda. Allow the mixture to stand for 5 minutes and dilute to 200 cc. A deep orange colour appears in B due to the formation of picramic acid from creatinine. The creatine in A gives no colour.

**228. Weyl's test for creatinine.** Treat 5 cc. with a few drops of a freshly prepared sodium nitroprusside and make the solution just alkaline with sodium hydroxide. A ruby-red colour appears. Boil. The solution turns yellow. Acidify with an excess of acetic acid and heat. A green tint appears, and a blue deposit of Prussian blue may result on standing.

**Purine bases.** These compounds are interesting because of their chemical relationship to uric acid. This relationship is shewn by the formulæ given on p. 62.

The purine bases found in meat extracts are chiefly hypoxanthine and xanthine. They can be obtained from the alcoholic solution obtained in Ex. 166, by evaporating off the alcohol, adding ammonia and precipitating with ammoniacal silver nitrate.

**Sarcolactic acid** is *dextro*- $\alpha$ -hydroxy-propionic acid.



The lactic acid found in muscle is *d*-lactic. That formed by the fermentation of lactose and other carbohydrates is generally *dl*-lactic. Certain bacteria, however, produce *l*-lactic acid (see p. 151).

Sarcolactic acid is present to a very small extent in fresh living muscle. The amount increases rapidly in fatigue, especially in the absence of a proper supply of oxygen. On leaving a fatigued muscle in an atmosphere of oxygen, the amount of lactic acid decreases.

There is a considerable production of lactic acid at the onset of rigor mortis. But if a fresh muscle be suddenly coagulated by dropping it into boiling water, there is no such marked production of the acid.

It is probable that the lactic acid appearing in fatigue and in rigor arises through the decomposition of some carbohydrate material in the muscle, but this has not been definitely established.

Sarcolactic acid is a liquid, soluble in water, alcohol and ether. It forms a characteristic zinc salt, which is obtained by boiling a solution with excess of zinc carbonate, filtering and evaporating slowly. The crystals contain two molecules of water of crystallisation, the zinc salt of ordinary fermentation lactic acid containing three.

229. **Hopkins' reaction for lactic acid.** To 3 drops of a 1 per cent. alcoholic solution of lactic acid in a clean, *dry* test tube add 5 cc. of concentrated sulphuric acid and 3 drops of a



saturated solution of copper sulphate. Mix and place the tube in a beaker of boiling water for about five minutes. Cool thoroughly under the tap, add two drops of a 0.2 per cent. alcoholic solution of thiophene, and shake. Replace the tube in the boiling water bath. As the mixture gets warm a fine cherry-red colour develops.

NOTE.—Lactic acid is oxidised in sulphuric acid solution to formaldehyde and acetaldehyde which react with thiophene in the presence of an excess of sulphuric acid to give a cherry-red colour. The copper sulphate aids this oxidation, which is inhibited by water.

**230. Uffelmann's reaction for lactic acid.** Treat a few cc. of Uffelmann's reagent with a few cc. of a dilute (0.4 per cent.) solution of lactic acid. The violet colour is instantly turned to a yellow.

NOTES.—1. Uffelmann's reagent is prepared by treating a 1 per cent. solution of phenol (carbolic acid) with very dilute ferric chloride till the solution becomes coloured an amethyst-violet.

2. The reaction is not very reliable, since other acids as tartaric, oxalic and citric give it.

**231. The Formation of Lactic Acid in Fatigue.** A pithed frog is kept on ice for about half-an-hour. Remove one hind limb and replace it on the ice. Expose the lumbar plexus of the other side and stimulate it electrically by means of a strong interrupted current for at least ten minutes. Cut off the hind limb, strip the skin off the two limbs and treat the muscles separately as follows: Rapidly remove the muscles, grind them with ice-cold 95 per cent. alcohol and sand. Transfer the mixture to a beaker, and warm for a few minutes on the water bath. Filter through a small paper and evaporate to complete dryness on a water bath. Treat the residue with about 5 cc. of cold water and rub it up thoroughly with a glass rod. Filter and boil the filtrate with as much animal charcoal as will lie on a threepenny piece. Filter and evaporate the filtrate to complete dryness on a water bath. Allow the residue to cool and apply Hopkins' test by treating the residue with strong sulphuric acid, shaking round till solution is obtained, transferring to a dry test-tube, adding three drops of saturated copper sulphate, etc. A fine red colour develops in the tube containing the extract from the tetanised muscle, but none or very little in the other.

**Glycogen.** The percentage of glycogen in fresh muscle varies from 0.5 to 1 per cent., so that the total amount in all the muscles of the body may be greater than in the liver. The muscle glycogen decreases after muscular exercise, but not so rapidly as that in the liver.

The estimation of glycogen is described on p. 123.

## CHAPTER VIII.

### THE COMPOSITION OF THE DIGESTIVE JUICES AND THE ACTION OF CERTAIN ENZYMES.

The digestive enzymes or ferments are bodies that have the power of accelerating the rate of hydrolysis of certain substances. They are divided into groups depending on the nature of the substance on which they act (the so-called substrate). Thus those acting on starch are called amylolytic; on proteins, proteolytic; on fats, lipolytic, etc. The enzymes are sometimes named in such a way as to indicate their origin and their action, the termination *-ase* being employed. Thus ptyalin, the amylolytic enzyme of saliva, can be termed salivary amylase, to distinguish it from pancreatic amylase (amyl-opsin). Gastric lipase, the lipolytic enzyme of the gastric juice, is similarly distinguished from pancreatic lipase (steapsin).

The chemical composition of the enzymes is at present uncertain, owing to the extreme difficulty of preparing them in a pure state. The proteolytic enzymes are either proteins, or compounds so readily adsorbed by proteins that it is impossible to separate them. The enzymes acting on certain of the carbohydrates are possibly themselves of a carbohydrate nature.

The properties of the enzymes as a class are as follows: they are soluble in water, dilute salt solutions, dilute alcohol and glycerol. They are precipitated by saturation with ammonium sulphate and by strong alcohol. They are readily carried down by different precipitates, probably by a process of adsorption. They are colloidal and non-diffusible. They are most active at a certain temperature,

called the optimum temperature, which is generally about  $45^{\circ}\text{C}$ . Their action is suspended by cooling, but is completely destroyed by raising the temperature to  $100^{\circ}\text{C}$ .

The enzymes are remarkably specific in their action, that is, they act only on a particular substance or on a group of substances having some similarity in chemical composition and configuration. A striking example of this is seen in the case of the glucosides (see page 103). The enzyme maltase ( $\alpha$ -glucose) hydrolyses  $\alpha$ -methyl- and  $\alpha$ -ethyl-*d*-glucosides, but has no action on  $\beta$ -methyl- or  $\beta$ -ethyl-*d*-glucosides, or on any *l*-glucoside or on *d*- or *l*-galactosides. The enzyme emulsin ( $\beta$ -glucose) acts only on  $\beta$ -ethyl, methyl or phenyl-*d*-glucosides. Lactase acts only on the  $\beta$ -galactosides. It is probable that the enzyme first unites with the substrate, and to do this it must have a configuration in space corresponding with that of the substrate. According to Bayliss the preliminary union of enzyme with substrate is a process of adsorption. Though adsorption phenomena are probably of great importance in enzyme action, this does not give any simple explanation of the remarkable specificity which is characteristic of enzyme action, but not of adsorption.

An important factor in the action of an enzyme is the concentration of hydrogen ions in the medium in which it acts. For each enzyme there is a particular hydrogen-ion concentration or  $P_{\text{H}}$  at which the velocity of reaction is greatest. This is the *optimum reaction* of that particular enzyme. The optimum  $P_{\text{H}}$  of certain enzymes is given on page 32. It is interesting to note that in the case of trypsin the optimum reaction seems to vary with the nature of the substrate, being  $P_{\text{H}} = 8.0$  when acting on fibrin and  $P_{\text{H}} = 6.7$  when acting on casein.\* Michaelis has made a special study of the significance of the optimum reaction, and claims that it is partly dependent on the effect

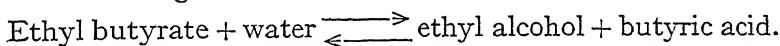
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\* The author has not been able to confirm this, finding  $P_{\text{H}} = 8.1$  the optimum reaction for the hydrolysis of casein by trypsin.

of the reaction on the nature of the dissociation of the enzyme, which in many cases functions as an ampholyte (see pages 11 and 31). At the iso-electric point of an ampholyte it exists mainly in the undissociated state. If the hydrogen-ion concentration be greater than at the iso-electric point, the enzyme is positively charged, that is, it exists mainly as kations : if the solution be alkaline to the iso-electric point, the enzyme exists mainly as anions, Michaelis has determined the iso-electric points of certain of the enzymes by various methods, and concludes that maltase, trypsin and erepsin are only active as anions ; pepsin as kations ; whilst invertase is only active as undissociated molecules. He makes the interesting suggestion that though undissociated pepsin has no action on ordinary proteins, yet it has the property of clotting milk, that is a rennetic action (see p. 208).

The action of most enzymes is retarded by the accumulation of the products of the reaction, and in certain cases the reaction is reversible.

This is well seen in the case of lipase, which induces the following reaction :—



The velocity of reaction is proportional to the amount of the enzyme present, provided that the amount of the enzyme is very small compared with that of the substrate. If the amounts of enzyme and substrate are at all comparable, the laws of mass action are followed. But complications are introduced by the fact that some of the enzyme is thrown out of action by being absorbed by the products of the action.

In certain cases enzyme action is dependent on the simultaneous presence of two substances. These are sometimes called co-ferments. It has been shewn that the zymase that is responsible for the alcoholic fermentation of sugar by yeast can only act in co-operation with phosphates and some substance that is diffusible and not

destroyed by boiling. Also the lipase of the pancreas requires the presence of some soluble, heat-stable substance to allow it to act. Bile salts have this property, as has been seen in a previous chapter. The action of the enzymes can be retarded by certain substances. These are of two classes: paralyzers and anti-enzymes. The paralyzers are generally salts of the heavy metals, which probably alter the physical state of the colloidal enzymes. The anti-enzymes are of an organic nature. They probably combine with the enzyme and thus prevent it from acting on the substrate. Examples are seen in the case of the anti-trypsin of normal serum, of the intestinal mucous membrane and of the tissues of intestinal parasitic worms.

### A. Saliva.

Saliva is of value as a lubricant in the act of deglutition, and in some animals this is its sole function.

232. Collect about 5 cc. of your own saliva in a small beaker. Test the reaction with neutral litmus paper: it is alkaline.

NOTE.—The first portion of saliva collected is very apt to be neutral, or even slightly acid, probably owing to bacterial decomposition in the mouth. But if the secretion is free, that collected later is invariably alkaline.

233. Transfer the saliva to a test-tube and add strong acetic acid. A stringy precipitate of mucin is formed, insoluble in excess of acid. Stir the mixture vigorously with a glass rod: the mucin forms a clump which can be removed by the rod. To the clear fluid remaining add some Millon's reagent and heat cautiously. Only a slight red precipitate is formed, showing that the proteins of saliva consist almost entirely of mucin.

### B. Ptyalin.

Ptyalin, or salivary amylase, is an enzyme that acts on boiled starch and certain other polysaccharides, the chief end product being the disaccharide maltose. It is possible that small amounts of glucose are also formed. It is claimed by certain workers that for the complete hydrolysis of starch three ferments are necessary, viz., *amylase*,

that converts starch into dextrins ; *dextrinase*, that converts dextrins into maltose ; and *maltase*, that converts maltose into glucose.

In the case of the action of ptyalin on starch as conducted *in vitro*, the final product consists of about 80 per cent. of maltose, the remaining 20 per cent. being a comparatively simple dextrin called "stable" dextrin, owing to its resistance to the further action of the enzyme. But if this dextrin be isolated the action of ptyalin is to hydrolyse it very slowly and incompletely to equal molecular parts of maltose and glucose.

The optimum reaction for ptyalin is at  $P_H = 6.7$ .\* The enzyme is rapidly destroyed should the reaction be markedly acid to this, as it is during full digestion in the stomach. But the presence of proteins, which function as buffers, prevent the hydrochloric acid secreted in the early stages of digestion from causing too high a concentration of hydrogen-ions for the action of the ptyalin. This, combined with the absence of active mechanical movements in the cardiac end of the stomach, allows salivary digestion to be carried on in the stomach for about 30 minutes after the ingestion of a mixed meal.

Ptyalin is remarkable in that it is inactive in the absence of electrolytes. As the author first demonstrated,† the influence of electrolytes on amylolytic action is dependent on the negative ion (kation). These have not all the same activating power, the chloridion being the most effective. A satisfactory explanation of this effect has not yet been advanced.

The optimum concentration of sodium chloride is between 0.02 per cent. and 2 per cent., between which limits very slight differences can be observed, but the difference between the action of the enzyme in a salt-free mixture and in one containing 0.02 per cent. of sodium

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\* It has recently been stated that the optimum reaction for malt diastase is at  $P_H = 4.9$ .

† *Journ. of Physiology*, 1903.

chloride is enormous. It is therefore of the utmost importance in all quantitative experiments on the action of the enzyme to ensure that about 0·1 per cent. of the salt is present. The beneficial effect of sodium chloride on salivary digestion offers a simple teleological explanation of the desire for salt when eating carbohydrate foods.

The estimation of ptyalin has been attempted by a variety of methods, none of which are very satisfactory. The most important of these methods are :

1. *Roberts' Achromic Point method.* A given amount of enzyme is added to a measured amount of starch paste at 40° C. Portions of the digest are treated with dilute iodine at intervals and the time when the iodine fails to give a colour is noted.

2. *Wohlgemuth's method.* A fixed amount of soluble starch is digested with varying amounts of the enzyme for a stated time. Iodine is added to each, and the amount of enzyme that just converts the whole of the starch to dextrin is determined.

3. *Reduction methods.* Starch is digested with the enzyme and the amount of maltose formed in a given time is determined.

C. Lovatt Evans (*Journal of Physiology*, xliv., p. 220) has criticised the various methods, and claims that the only correct method is a reduction method carried out under certain defined conditions of starch concentration, temperature, etc. His main contention is that the amount of maltose formed is only proportional to the amount of enzyme added, provided that

1. Less than 30 per cent. of the starch has been converted, at which stage a blue reaction is still obtained with iodine.

2. The digestion period is a short one (ten minutes).

3. The concentration of the starch is about 3 per cent.



His objection to the Achromic Point method is that the end point is very difficult to determine, especially with weak enzymes, and that the digestion ("chromic") period bears no simple relationship to the concentration of the enzyme. This is undoubtedly well-founded, for if the enzyme be halved the chromic period is more than doubled.

His objection to Wohlgemuth's method is mainly based on the fact that in certain experiments the amounts of enzyme added are in a geometrical series, and so the only results obtainable will also be in a geometrical progression. But this is a poor argument, for the experimenter can vary the amount of enzyme added as he pleases.

It is curious that neither Wohlgemuth nor Lovatt Evans take any serious precautions to maintain the optimum  $P_H$  or salt content. In fact, certain results of the latter with the Achromic method may be due in part to the dilution of the chlorides of the saliva and not merely to the dilution of the enzyme.

After a considerable amount of investigation the author has decided to adopt a colorimetric method for ordinary work (see Ex. 242). It is by no means perfect, but it is fairly rapid, and the end point is more easily determined than in the Achromic or Wohlgemuth's method. The relationship between amount of enzyme and rate of digestion is fairly exact for moderate changes in dilution.

234. Obtain diluted saliva as follows: Warm some distilled water in a beaker to about  $40^{\circ}\text{C}$ . With a portion of this thoroughly rinse out the mouth. Now take about 20 cc. of the warm water into the mouth and move it about by the tongue for at least a minute. Collect the fluid thus obtained in a clean beaker, and repeat the process once or twice, depending on the amount required. Transfer the whole to a flask, close with the thumb, shake vigorously, and filter.

235. In a clean test-tube place 5 cc. of 1 per cent. starch paste, freshly prepared with distilled water (see Ex. 135) and 5 cc. of the diluted saliva. Mix well, place a glass tube or pipette in the tube, and place the test-tube in a water bath maintained at about 40° C. Place a series of drops of iodine solution (about 0.02 N.) on a clean, dry, white porcelain or opal glass plate. From time to time allow a drop of the digesting mixture to fall on to one of the drops of iodine, taking care that the iodine is not transferred to the digesting mixture. A blue colour should be produced at first, but with the drops added later the colour becomes blue-violet, red-violet, red-brown, light brown, and finally no increase of colour is obtained. If no increase of colour is obtained with the first drop, repeat the experiment, making the first test immediately after the starch and saliva have been mixed. If the blue colour persists for a long time, try the effect of adding a couple of drops of 5 per cent. sodium chloride to the digesting mixture.

When a drop of the mixture fails to give a colour with the iodine, boil a few cc. with a little Fehling's solution. A well-marked reduction is obtained, showing that the enzyme ptyalin has converted the starch into a reducing sugar, which is, however, not glucose, but maltose.

236. Perform a control test by first boiling and then cooling the saliva before adding it to the starch. The colour with iodine is always blue, and the solution does not reduce Fehling's.

237. **Achromic Point method.** Measure 5 cc. of 1 per cent. soluble starch (see p. 391) into a test-tube. Add 2 cc. of a buffer solution of  $P_H = 6.7$  and 2 cc. of 0.5 per cent. sodium chloride. Place the tube in a water bath maintained at 38° to 40° C. for a few minutes. Have ready a series of test-tubes, each containing about 3 cc. of distilled water. To each tube add a couple of drops of dilute iodine (about 0.01 N.). To the tube containing the warmed starch add 2 cc. of the diluted saliva, mix well, and note the time. At intervals transfer a drop or two of the digesting mixture to one of the tubes containing the diluted iodine by means of a quill tube, and shake. The colour obtained is blue at first. Determine the time when the addition ceases to produce any colour. This point, which is the moment when the last trace of erythro-dextrin is converted

to achroo-dextrin and maltose, is known as the achromic point. The time that is taken to reach this point ("chromic period") is a measure of the activity of the ferment.

If the chromic period is less than 4 minutes, dilute the enzyme with one or more volumes of distilled water and repeat the experiment.

NOTES.—1. The buffer solution is added to maintain the optimum concentration of hydrogen ions. It is prepared by treating 50 cc. of 0.2 M. acid potassium phosphate with 21 cc. of 0.2 N. soda and adding distilled water to make a total volume of 200 cc. (See p. 27.)

2. The reason for the addition of the sodium chloride is explained in the text on p. 188.

3. Starch paste can be used instead of soluble starch, but it is difficult to measure accurately by means of a pipette, owing to its viscosity.

4. The use of the author's "Distributor" (fig. 29) for automatically delivering a given volume of fluid is convenient for measuring about 3 cc. of water into each tube. Distilled water must be used, owing to the action of tap water on iodine. The iodine should not be added until just before the addition of the digesting mixture.

238. Repeat the above experiment, substituting 2 cc. of distilled water for the 2 cc. of sodium chloride. The chromic period is much prolonged.

239. Determine the effect of dilution of the enzyme on the chromic period. It will be found that the chromic period lengthens out unduly as the ferment is diluted; that is, the period is more than trebled by a three-times dilution.

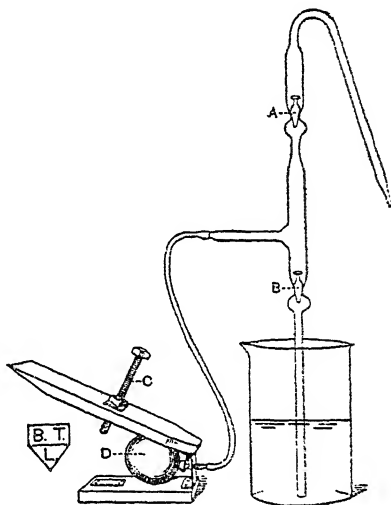


Fig. 29. Cole's "Distributor" for the automatic delivery of a given volume of fluid.

The fluid is placed in the beaker and the air is driven out of the apparatus by repeatedly compressing the rubber ball. On firmly pressing down the hinged board a given volume of fluid is driven out of the apparatus. The volume delivered can be varied by adjusting the screw C. The rubber tubing should be thick pressure tubing and as short as possible. The wooden base should be screwed down.

240. Determine the effect of change of reaction on the chromic period. It will be found that it is least at  $P_H = 6.7$ , lengthening out as the solution is made acid or alkaline to this. With small changes of reaction, however, the activity is but slightly affected. Determine the chromic period at  $P_H = 5.7$  and  $7.7$ .

241. **Wohlgemuth's method.** Number a series of clean dry test-tubes 1 to 10.

Into 1 and 2 measure 1 cc. of the diluted enzyme.

Into 2 to 10 measure 1 cc. of distilled water.

Mix the contents of 2 and transfer 1 cc. from 2 to 3.

Mix the contents of 3 and transfer 1 cc. from 3 to 4.

Continue in this way, rejecting 1 cc. of the fluid from 10.

To each tube add 5 cc. of 1 per cent. soluble starch (see Note 2), commencing with tube 10. Mix the contents of the tubes and place them in a water bath at  $40^\circ \text{C}$ ., noting the time when they are placed in the bath. Allow the tubes to remain in the bath for exactly 30 mins. Remove the tubes and immerse them all in cold water to stop the action.

Arrange them in order in a rack.

To each tube add cold distilled water to about 2 finger-breadths from the top of the tube. Now add 3 drops of 0.02 N. iodine to each tube and mix, commencing with the tube 1. This will probably give no colour, whilst tube 10 will probably give a deep blue. Between these limits tubes will probably be found which are red and purple. Select the tube with the lowest number that has a blue tinge mixed with the red. In the tube numbered one below this there has been enough enzyme to completely convert 5 cc. of soluble starch to erythrodextrin in 30 minutes.

Wohlgemuth indicates the concentration of enzyme in the following way. He determines the volume of 1 per cent. starch that would be converted to erythrodextrin by 1 cc. of the enzyme solution in 30 minutes. He calls this the "diastatic power" of the solution, and indicates it by  $D$  for 1 per cent. and by  $d$  for 0.1 per cent. starch. He also indicates the temperature and period of digestion. Thus in the above experiment, if the 6th tube were blue-violet and the 5th tube were red, then since the 5th tube contains  $\frac{1}{16}$  cc. of the enzyme, and this amount has converted 5 cc.

of 1 per cent. soluble starch to erythro-dextrin in 30 minutes, then 1 cc. would convert  $16 \times 5 = 80$  cc. of starch. If the temperature of the bath is  $40^{\circ}$  C., then  $D \frac{40^{\circ}}{30} = 80$ .

NOTES.—1. In the experiment as conducted above, the possible error is nearly 100 per cent. A nearer approximation can be made by repeating the experiment with more gradual dilutions that will depend on the result obtained. Thus, if  $\frac{1}{16}$  cc. gives a red and  $\frac{1}{32}$  cc. gives a violet, D may be anything between 80 and 160. To 1 cc. of the enzyme add 15 cc. of distilled water, mix well, and measure 1, 0.9, 0.8, 0.7, 0.6 and 0.5 cc. into a series of 6 tubes. Make up the volume to 1 cc. in each case by the addition of distilled water. Add 5 cc. of the soluble starch and repeat the experiment. One of the following values for D will then be obtained: 80, 88, 100, 114, 133, or 160.

2. Though in Wohlgemuth's original method the instructions are to use pure 1 per cent. soluble starch, the author finds that the results obtained are much more reliable if the hydrogen-ion concentration and the salt content be maintained at the optimum. The starch solution is prepared by treating 100 cc. of 2 per cent. soluble starch (see p. 391) with 25 cc. of a buffer solution of  $P_H = 6.7$  (see Note 1, Ex. 237), 25 cc. of 1 per cent. sodium chloride and 50 cc. of distilled water. The starch solution should be freshly prepared.

3. It is important to add exactly the same amount of iodine to each tube in all the experiments. The iodine should be measured by means of a dropping pipette (see fig. 5).

242. **The Method of "the first change."** Measure 10 cc. of 2 per cent. soluble starch (see p. 391) into a test-tube. Add 2 cc. of a buffer solution of  $P_H = 6.7$  (see Note 1, Ex. 237) and 2 cc. of 0.5 per cent. sodium chloride. Place the tube in a water bath at  $40^{\circ}$  C. for some minutes until it has attained the bath temperature. Have ready a series of tubes containing 3 cc. of distilled water, to each of which has been added a single drop of 0.01 N. iodine by means of a dropping pipette (fig. 5). Now add 2 cc. of the enzyme to the starch tube by means of a 2 cc. pipette, discharging this by blowing through it for a second into the solution. Note the time of the addition of the enzyme. Seal the tube by the thumb and mix by shaking; immediately replace the tube in the water bath. Insert a quill tube. At the end of 1 minute allow a single drop of the digesting mixture to fall into one of the iodine tubes, shake by mixing, and place this tube in the first hole of a test-tube rack. At the end of 2 minutes, allow another drop to fall into another iodine tube, shake and place this in the second hole of the rack. Continue in this way until the colour given by the digestion mixture is violet. Now examine the tubes carefully in diffuse daylight, and select the tube in which

the pure "starch-blue" colour has just definitely changed and a very slight tinge of violet is perceptible. From the position of this tube in the rack the time required to produce this effect is obtained. It is a measure of the activity of the enzyme.

If the digestion time is much less than 10 minutes, repeat the experiment with a diluted enzyme, attempting to arrive at a digestion period of about 10 minutes. Thus, if in the first experiment the third tube shows the change, dilute 3 cc. of the enzyme with 7 cc. of distilled water. If the fourth tube shows the change, dilute 4 cc. with 6 cc. of distilled water, and so on. In the second experiment it is advisable to take a sample drop every half-minute when near the expected end point. These "half-minute" tubes can be subsequently identified by placing them behind the "minute" tubes in a double test-tube rack.

NOTES.—1. The drops of iodine should not be added to the distilled water in the tubes until just before (or even after) the enzyme has been added to the starch tube. It is important to add only one drop to each tube.

2. The author's "Distributor" (fig. 29) is convenient for measuring the 3 cc. of distilled water.

3. The effect of the concentration of hydrogen-ions can be investigated by varying the  $P_H$  of the buffer solution added (see Ex. 240).

4. The author suggests that the unit of amylase should be taken as the amount which can convert 10 cc. of 2 per cent. soluble starch to the stage obtained in 10 minutes. So if 2 cc. of a 4 in 10 dilution effects the change in 9.5 mins., then 2 cc. contains  $\frac{10}{4} \times \frac{10}{9.5} = 2.62$  units. This can be expressed,  $A = 131$  per 100 cc.

### C. Gastric Juice.

Human gastric juice has been obtained in certain cases in which an artificial opening into the stomach has been necessitated owing to stricture of the oesophagus. It contains about 0.37 per cent. of hydrochloric acid, small amounts of the chlorides of sodium, calcium and potassium, traces of phosphates and of mucin, together with the enzymes, pepsin, rennin and lipase.

From a practical standpoint the composition of the gastric contents at stated times after the ingestion of

standard test meals is more important than that of the juice as actually secreted. The hydrochloric acid, secreted mainly by the oxyntic cells of the fundus, neutralises the alkaline salts of the saliva to form sodium chloride. It also combines with the proteins of the saliva and of the food to form acid-protein compounds. These function as weak acids. In this way the percentage amount of free, uncombined hydrochloric acid in the gastric contents may be very considerably less than in the naturally secreted juice. Further alterations are brought about by the fermentation of the carbohydrates to lactic acid. This is mainly caused by certain organisms called *sarcinae*, which are very commonly present in the stomach. If the starchy foods are thoroughly masticated and mixed with the saliva, the polysaccharides are rapidly broken down by the ptyalin to maltose. This is passed through the pylorus and leaves little for the *sarcinae* to ferment. The presence of much lactic acid generally indicates a diminished secretion of hydrochloric acid, which inhibits the action of the lower organisms.

*Ewald test meal.* The patient under investigation is starved for at least 12 hours. The meal consists of 400 cc. of weak tea, without milk or sugar, and 50 grams. of dry toast, which should be well masticated. After 1 hour the gastric contents are removed by syphonage through a soft rubber stomach tube.

The fluid obtained is measured and filtered. The normal physiological volume is between 40 and 70 cc. A marked increase in volume probably indicates motor insufficiency of the stomach, or hypersecretion, the latter generally being associated with an increased amount of hydrochloric acid (hyperchloridia).

*Chemical examination.* The reaction of the filtered juice is nearly always acid to litmus paper. The percentage amount of the following substances must be determined ;

- A. Total acidity. (Ex. 243.)
- B. Total chlorides. (Ex. 244.)
- C. Mineral chlorides. (Ex. 245.)
- D. Active hydrochloric acid. (B - C.)
- E. Free hydrochloric acid. (Ex. 246.)
- F. Combined hydrochloric acid. (D - E.)
- G. Abnormal acidity. (A - D.)

It is usual to express all these results in terms of grams. of hydrochloric acid per cent.

*Total acidity* is determined by titration with 0.1 N. soda, the indicator being phenol-phthalein.

*Total chlorides* is obtained by adding 1 cc. of a saturated solution of sodium carbonate to 100 cc. of the filtrate, evaporating on the water bath, heating to a dull red heat and estimating the total chlorides by Volhard's method.

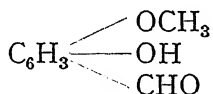
*Mineral chlorides* are determined in a similar way, except that the sodium carbonate is not added. Most of the free hydrochloric acid is evolved on heating to dryness. On incineration the protein matter is destroyed and any hydrochloric acid combined with it is evolved. The only chloride left is that which was originally present in the form of non-volatile chlorides of sodium, etc. The difference between this and the former estimation is a measure of free hydrochloric acid plus that combined with proteins, the sum of these being known as "active hydrochloric acid." Since active hydrochloric titrates with soda to phenol phthalein, the difference between total acidity and active hydrochloric is a measure of the abnormal acids present, generally lactic acid.

In a few cases the author has observed that the active hydrochloric acid is greater than total acidity. This puzzling result is probably due to the presence of small amounts of ammonium chloride, which is volatile at a low red heat, and would be removed in the estimation of mineral chlorides. After treatment with sodium carbonate, the ammonium chloride would be converted to sodium chloride. In such a case, the mineral chlorides work out too low and the active hydrochloric proportionally too high, and in the absence of lactic acid the active hydrochloric acid will exceed the total acidity.

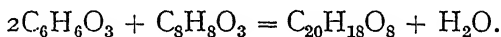
*Free hydrochloric acid.* The only satisfactory clinical method for the estimation of this is by use of Gunzberg's



reagent. This is an alcoholic solution of phloroglucin (symmetrical tri-hydroxy benzene,  $C_6H_3(OH)_3$  and vanillin



When evaporated with hydrochloric acid on the water bath these condense to form a brilliant red compound,  $C_{20}H_{18}O_8$ .



In the absence of free hydrochloric acid only a brown colour is obtained. A brown colour also develops in the presence of hydrochloric acid if the mixture be overheated.

There are two methods of applying the test. One is to dilute the solution until it fails to give a positive reaction, assuming that it is just positive with 0.0004 N.HCl (0.00146 per cent.). The other, and better, method is to titrate a measured sample with soda until a drop of the titrated mixture just fails to give the reaction.

From comparisons with the electrical method of measuring hydrogen-ion concentration (p. 19) it would seem that the estimation by Gunzberg's reagent is accurate when the acidity is high or moderate, but that it gives too low a result with gastric contents relatively deficient in hydrochloric acid.

At one time it was claimed that Toepfer's reagent (p. 22) only reacted with free hydrochloric acid. It has now been shown that both this indicator and also congo red react with lactic acid if this be sufficiently concentrated, and that the amount of free hydrochloric acid as determined by their use may be far in excess of the amount actually present.

*Results of analysis.* In normal cases the following may be taken as the limits, the results being expressed in grams. of HCl per cent.

|                      |    |              |
|----------------------|----|--------------|
| Total acidity ..     | .. | 0.14 to 0.26 |
| Total chlorides ..   | .. | 0.2 to 0.3   |
| Mineral chlorides .. | .. | 0.09 to 0.12 |
| Active HCl ..        | .. | 0.14 to 0.26 |
| Free HCl ..          | .. | 0.07 to 0.15 |

G. Graham [*Quarterly Journal of Medicine*, IV., p. 315 (1911)], has published an interesting series of cases. The average results he obtained are as follows :

|                           | Total<br>Chlorides. | Mineral<br>Chlorides. | Active<br>HCl. | Ratio<br>Active: Mineral. |
|---------------------------|---------------------|-----------------------|----------------|---------------------------|
| Gastric ulcer ..          | 0.335               | 0.099                 | 0.236          | 238 : 100                 |
| Dilated stomach ..        | 0.256               | 0.094                 | 0.182          | 194 : 100                 |
| Carcinoma of<br>stomach.. | 0.197               | 0.142                 | 0.052          | 37 : 100                  |

It will be noted that in carcinoma of the stomach the ratio of active HCl to mineral chlorides is markedly sub-normal, due to a decreased secretion of HCl and the neutralisation of a good deal of this by some alkaline secretion. This change would seem to be more diagnostic than the absence of free HCl, since the latter occurs in certain other pathological conditions. In gastric and duodenal ulcer there is an increase in the amount of free and active HCl, and the active mineral ratio is apt to be above normal.

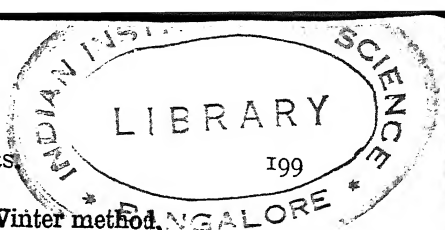
*Mixture for analysis.* The following mixture can be analysed for the purpose of acquiring the necessary technique :—

|                                |    |         |
|--------------------------------|----|---------|
| 1 per cent. sodium chloride .. | .. | 14 cc.  |
| 0.1 N. HCl ..                  | .. | 50 cc.  |
| 2 per cent. peptone ..         | .. | 26 cc.  |
| Distilled water to make ..     | .. | 100 cc. |

243. **Total acidity.** To 10 cc. add 4 drops of a 0.5 per cent. solution of phenol phthalein in 50 per cent. alcohol. Titrate with 0.1 N. soda until a faint but definite pink tinge is obtained. The end point is not very sharp, owing to the buffer action of the peptone.

*Calculation.* 1 cc. of 0.1 N. NaOH = 0.00365 gram. HCl.

Express the result in grams. of HCl per 100 cc.



## 244. Total Chlorides by the Prout-Winter method.

*Principle of Volhard's process for the estimation of chlorides.* A measured amount of standard silver nitrate is added to a known amount of the solution, or to a properly prepared extract of a known amount of material. The amount of silver used must be more than enough to completely precipitate the whole of the chlorides. Iron alum is added (to serve as an indicator), the mixture is made acid with nitric acid (to prevent the precipitation of other substances), and the mixture made up to a definite volume. The precipitate of silver chloride is filtered off and the amount of silver in an aliquot portion of the filtrate determined by titration with standard thiocyanate solution. From the amounts of standard silver originally used and that found in the filtrate the amount precipitated by the chlorides in the material taken can be calculated.

*Solutions required.* 0.1 N. Silver nitrate. Dissolve 16.99 grams. of pure fused silver nitrate in distilled water and make the volume up to 1 litre. The solution should be kept in the dark.

1 cc. = 0.00365 gram. HCl.

0.1 N. thiocyanate. Dissolve about 15 grams. of potassium, or about 10 grams. of ammonium thiocyanate, in a litre of distilled water and mix thoroughly. Standardise this in the following way:—Measure out 20 cc. of the standard silver nitrate into a 150 cc. beaker or Erlenmeyer flask. Add about 60 cc. of distilled water, 5 cc. of pure nitric acid, and 5 cc. of a cold saturated solution of iron alum. Titrate with the thiocyanate from a burette. A white precipitate of silver thiocyanate is formed. Continue to add the thiocyanate until a faint permanent pink (due to ferric thiocyanate) is obtained. Let  $x$  cc. be the amount of thiocyanate required. To 1 litre of the solution add  $\frac{1000(20-x)}{x}$  cc. of distilled water. The mixture should now be 0.1 N. It

is advisable to check the strength once more against the standard silver.

*Iron alum.* A cold saturated solution in distilled water.

*Pure nitric acid*, free from chlorides.

**Method.** To 10 cc. of the filtered fluid in a porcelain or silica crucible (not in an evaporating basin) add 1 cc. of a saturated solution of sodium carbonate. Evaporate to complete dryness on a boiling water bath. Support the crucible on a pipe clay triangle, and cautiously heat with a Bunsen burner. Gradually raise the temperature until the mass has completely carbonised. The heating should be continued until as much as possible of the carbon has disappeared and the whole has been raised to a dull red heat. Remove the flame and allow the crucible to cool until it can be handled. Add about 10 cc. of distilled water and carefully stir with a glass rod. Pour the fluid, together with the little pieces of carbon, into a 100 cc. graduated flask, using a small funnel. Repeat the extraction with another 10 cc. of water: then with 5 cc. of pure nitric acid and 5 cc. of water: then twice more with water. Wash

down the funnel with a little water and remove it. To the flask add 20 cc. of the standard silver nitrate, measuring this by means of a pipette. Then add 5 cc. of the iron alum, and make the volume up to the mark with distilled water. Mix thoroughly and filter through a dry paper into a clean, dry, 100 cc. measuring cylinder. Collect at least 90 cc. of the fluid and note its exact volume. Transfer it to a beaker or flask, wash out the cylinder with a little water and titrate with the thiocyanate from a burette until a faint but permanent pink colour is obtained.

*Calculation.* Suppose that 92 cc. of the filtrate were taken, and that this required 11.4 cc. of thiocyanate.

Then 100 cc. of the filtrate would require  $11.4 \times \frac{100}{92} = 12.4$  cc.

So the chlorides have precipitated  $20 - 12.4 = 7.6$  cc. of the standard  $\text{AgNO}_3$ .

So total chlorides in 10 cc. =  $7.6 \times 0.00365$  gram. HCl.

So total chlorides in 100 cc. = 0.277 gram. HCl.

**245. Mineral Chlorides.** Repeat the above exercise in every particular, except that the sodium carbonate is not added. The calculation is made in the same way as in the above case.

**Active HCl** is the difference between these two results. It should be compared with the result of Ex. 243.

**246. Free Hydrochloric acid.** To 10 cc. of the filtered fluid in a small beaker add 1 cc. of 0.1 N. sodium hydroxide from a burette. Stir well and place a drop of the mixture, together with a drop of freshly prepared Gunzberg's reagent (see p. 390), into a white evaporating basin. Heat on a boiling water bath. If free HCl is still present the film will develop a brilliant carmine tinge as it dries. In that case add another cc. of the NaOH, and repeat the evaporation with another drop of the reagent. Proceed in this way, adding 1 cc. of soda at a time until a negative test is obtained. Then add 0.5 or less of 0.1 N. HCl from a burette and repeat the test. If it is now positive add 0.1 or 0.2 cc. of soda and repeat again. It is necessary to determine the amount of soda and HCl that must be

added so that the test is just negative. With a little experience one can judge how near one is to the end point, from the rapidity with which the red colour develops and from the intensity obtained.

*Calculation.*  $x$  cc. of soda, together with  $y$  cc. of HCl, just fail to give a positive reaction.

$(x - y)$  cc. 0.1 N. soda are required to neutralise the free HCl in 10 cc. of the fluid.

Free HCl =  $(x - y) \times 0.0365$  gram. per cent.

#### D. Pepsin.

Pepsin is the proteolytic enzyme secreted by the chief or peptic cells of the gastric glands. These cells elaborate the zymogen, pepsinogen, which is converted to pepsin by hydrochloric acid.

Pepsin is remarkable in only acting in a decidedly acid medium, the optimum  $P_H$  being about 1.4. Not only is it inactive in neutral or alkaline solutions, but the latter rapidly destroy it. Pepsinogen is much more stable to alkaline solutions, and can thus be distinguished from pepsin. Acids other than hydrochloric can be employed in artificial digestion experiments, but not so successfully. With such weak acids as acetic and butyric the digestive action, even in 4 per cent. of the acid, is very feeble, since the requisite hydrogen-ion concentration cannot be obtained. Neutral salts inhibit the action of pepsin, this being in marked contrast to the influence of sodium chloride on ptyalin.

The chemical nature of pepsin has not yet been established, it being almost impossible to separate it from substances on to which it is adsorbed. It may eventually transpire that the fact that all preparations contain iron and chlorine may not be without significance.

Commercial pepsin is prepared by extracting the mucous membrane of hogs' stomachs with dilute hydrochloric acid, filtering, and concentrating under reduced pressure at low temperatures, a large surface for evaporation being provided. A more active product can be

obtained by extracting the washed and minced mucosa with 3 parts by weight of 5 per cent. alcohol for 4 hours, filtering and concentrating under reduced pressure.

The products of action of pepsin differ with the nature of the protein undergoing digestion. The distinguishing feature of peptic digestion is that the final products consist mainly of simple proteins called peptones and polypeptides. It is true that in artificial digestions over long periods, traces of amino-acids are produced. But this action is of little physiological importance compared to the corresponding action of trypsin and erepsin. An account of some of the products formed during the peptic digestion of fibrin is given on p. 53.

It is possible that pepsin does not break the ordinary peptide linkage (see Note 5, Ex. 24). It is significant that it has no action on any artificial polypeptide, many of which are split into their constituent amino-acids by trypsin or erepsin. The proteoses and peptones formed by peptic digestion may be united in the intact protein molecule by some special form of linkage which is readily attacked by pepsin. This has not yet been satisfactorily demonstrated, but from various considerations it would seem to be highly probable.

Pepsin can be estimated by a variety of methods. A very well-known method is that of Mett. Egg-white is drawn up into fine glass tubes and coagulated by heat. Lengths are cut off, immersed in the solution for a stated time, and the amount of egg-white digested determined by measurement. Experience has shown that the length of egg-white digested varies as the square root of the amount of pepsin present, this relationship being known as the "Schütz-Borissow law." The preparation of satisfactory tubes is not a simple matter. J. Christiansen (*Biochem. Zeitschrift*, XLVI., p. 257) has described a method of standardising the egg tubes, but, perhaps in spite of this, the method seems to be falling into disuse.

The edestin method of Fuld (Ex. 250) is reliable, except

for solutions containing small amounts of pepsin and relatively large amounts of salts and other substances. These partially precipitate the edestin and render it impossible to make satisfactory observations.

For clinical purposes the author suggests that the simplest method of estimating pepsin would be to determine the time required for the clotting of "calcified" milk. It is true that the method would not distinguish pepsin from rennin, but it is improbable that rennin is secreted by the adult human stomach (see p. 207).

For the following experiments use a 0.5 per cent. solution of commercial pepsin (Armour's) in water.

247. Place equal amounts of fresh washed fibrin in four test-tubes labelled A, B, C, and D.

To A add 5 cc. of pepsin and 5 cc. of 0.4 per cent. HCl.

To B add 5 cc. of pepsin and 5 cc. of water.

To C add 5 cc. of water and 5 cc. of 0.4 per cent. HCl.

To D add 5 cc. of pepsin that has been boiled and then cooled, and 5 cc. of 0.4 per cent. HCl.

Place the four tubes in a water bath at 40° C. for at least thirty minutes.

*Note that in*

A, the fibrin swells up, becomes transparent, and then dissolves ;

B, the fibrin is unaltered ;

C, the fibrin swells up, becomes transparent, but does not dissolve ;

D, the fibrin is like that in C.

NOTE.—These exercises show that neither 0.2 per cent. HCl alone, nor pepsin alone, can digest fibrin, but that pepsin in the presence of 0.2 per cent. HCl has this property. In D the ferment pepsin has been destroyed by boiling. Fibrin is obtained by whipping blood at a slaughter house with a bundle of twigs, feathers, etc. The blood must be whipped as soon as it is shed. The mass of fibrin is placed on a sieve and thoroughly washed in running water to remove the haemoglobin. It is then chopped up on a board into small pieces.

248. **The detection of pepsin.** Obtain some fibrin that has been stained with carmine (see Note below). Treat the ferment solution with the same volume of 0.4 per cent. HCl. Divide this into two equal portions and label them A and B. Boil B for a minute, and cool the tube. To each tube add a few flakes of the stained fibrin. Place them on the warm bath for ten minutes. Shake and observe the colour of the fluid. In A it will be red. In B it will be almost or quite colourless.

NOTE.—The carmine solution for staining fibrin is prepared by dissolving 1 gram. of carmine in about 1 cc. of ammonia and adding 400 cc. of water. The solution is kept in a loosely-stoppered bottle till the smell of ammonia has become faint. Fresh washed fibrin is chopped finely, placed in the carmine solution for twenty-four hours, strained off and washed in running water till the washings are colourless. If not required immediately, it should be kept under ether and washed with water before use. It cannot be used for testing for trypsin, owing to the solubility of the dye in alkalies.

249. *Destruction of pepsin by alkalies.* To 5 cc. of the pepsin solution add 1 per cent. caustic soda drop by drop until the reaction is just faintly alkaline to litmus paper. Place in the warm bath for 10 minutes. Make the reaction just acid to litmus by the addition of 0.4 per cent. hydrochloric acid, and then add to the mixture its own volume of the same acid. Add some carmine fibrin and place the tube in the warm bath. The fibrin does not dissolve owing to the destruction of the pepsin by the alkali.

NOTE.—In light of the above experiment, it is unnecessary to test an alkaline solution for pepsin.

## 250. The estimation of Pepsin by Fuld's method.

**Principle.** An acid solution of edestin (the protein of hemp seeds) is precipitated by sodium chloride: the peptic digestion products are not precipitated.

### Solutions required.

1. Hydrochloric acid. Dilute 30 cc. of N/10 HCl to 100 cc. with distilled water.
2. 0.1 per cent. solution of edestin. Dissolve 0.1 gram. of pure edestin in 100 cc. of the hydrochloric acid at boiling point. Cool and make up to 100 cc. with the hydrochloric acid. If the solution is not clear it must be filtered.
3. Saturated (33 per cent.) solution of sodium chloride.

**Method.** Number a series of clean tubes from 1 to 10. Into



tubes 2 to 10 measure 1 cc. of the hydrochloric acid. Into tubes 1 and 2 measure 1 cc. of the gastric juice. Mix the fluid in tube 2 and transfer 1 cc. to tube 3. Mix this and transfer 1 cc. of the mixture to tube 4. Proceed in this way till each tube contains 1 cc. of fluid and each tube contains one-half of the amount of enzyme present in the tube with the next lower number, 1 cc. of tube 10 being rejected. To each tube add 2 cc. of the edestin solution. Mix and allow the tubes to stand at room temperature (15 to 17° C.) for 30 minutes. To each tube add 10 drops of the sodium chloride. The tubes with low numbers are probably clear, whilst the tubes with high numbers are cloudy. Note the tube with the lowest number that shows a cloud. The tube with the number next below it has an amount of gastric juice that just digests 2 cc. of the edestin in 30 minutes. Thus the number of cc. of edestin digested by 1 cc. of gastric juice can be calculated.

This is best denoted by  $pe \frac{16^\circ}{30'}$ .

Thus if tube 6 shows a cloud, then in tube 5 (containing 1-16th cc. gastric juice) digestion is complete. Supposing the temperature is 16° C., then  $pe \frac{16^\circ}{30'} = \frac{2}{16} = 32$ .

### 251. The estimation of Pepsin by Mett's method.

*Preparation of the tubes.* The whites of several new-laid eggs are beaten to break the membranes, strained through linen or muslin and allowed to stand till free from air bubbles. The liquid is then drawn up into lengths of glass tubing with an internal diameter of between 1 and 2 mm. Each length is laid flat on a piece of wire gauze, so arranged that it can be dropped into a saucepan of hot water, having a double bottom ("porridge saucepan"). The water in the saucepan is boiled and allowed to stand till that in the inner vessel has cooled to 85° C. The gauze with the prepared tubes is then placed in this inner vessel, and allowed to stand till the water is quite cold. The tubes can be preserved by sealing the ends with shellac.

*Method of estimation.* Cut off lengths of 2 cms., breaking the tubes sharply to get an even edge of coagulated egg-white.

Measure 10 to 20 cc. of the ferment into a small Erlenmeyer flask. In it place two of the tubes of egg-white, shake and cork, and place the flask in a thermostat at 40° C. for 24 hours. The mixture must not be shaken during the digestion. Measure the length of the tube (T) and of the remaining egg-white (W) by means

of a millimetre scale and a magnifying glass.  $T - W$  = the amount of protein digested (D). Take the average for the two tubes. D varies as the square root of the amount of ferment present.

NOTES.—Filtered gastric contents should be diluted with  $\frac{N}{30}$  HCl in the proportion of 1 cc. of gastric contents to 15 cc. of acid.

For practice use a 0.5 per cent. solution of commercial pepsin in  $\frac{N}{30}$  HCl.

Dilute 1, 4, and 9 cc. to 16 cc. with  $\frac{N}{30}$  HCl. The amounts of egg-white digested should be as  $\sqrt{1} : \sqrt{4} : \sqrt{9}$  i.e. as 1 : 2 : 3.

## 252. The estimation of Pepsin by Calcified Milk.

*Preparation of the milk.* To 50 cc. of fresh milk add 10 cc. of N. calcium chloride (5.55 per cent.), and make the volume up to 100 cc. with distilled water.

*Method.* Measure 5 cc. of the calcified milk into 4 or 5 rather wide test-tubes and place them in the warm bath at 38° C. Add 1 cc. of the enzyme solution to one of the tubes, mix by placing the thumb on the top of the tube and giving one shake; immediately replace the tube in the bath and note the time. Examine the tube at intervals, and note the time when a precipitate appears. With a 0.5 per cent. solution of commercial pepsin it is almost instantaneous. If the clotting time is very short, dilute 1 cc. of the enzyme with 9 or 19 cc. of distilled water; mix well, wash out the pipette, and repeat the test. Continue to make further dilutions of the solution thus obtained until the clotting time is between  $1\frac{1}{2}$  and  $1\frac{3}{4}$  minutes.

*Calculation.* The suggested unit is the amount of pepsin that will clot 5 cc. of the calcified milk in 100 seconds. The clotting time is inversely proportional to the amount of enzyme present.

Thus, suppose 1 cc. of a 1 in 120 dilution clots in 85 seconds, then 1 cc. of the original solution contains

$$120 \times \frac{100}{85} = 141 \text{ units.}$$

NOTE.—It is convenient to use a deep water bath with the bulb of an electric lamp dipping into the water. The tubes are contained in a wire basket fastened to the inner side of the bath. After the enzyme has been added, the tube is held in a sloping position and gently rotated backwards and forwards. The formation of the precipitate can be readily detected in the light from the

lamp. In this way the temperature of the mixture can be maintained constant, an impossibility when the tube has to be repeatedly removed for inspection. A stop-watch is an added convenience.

### E. Rennin and the Clotting of Milk.

When warm milk is treated with a neutral or faintly acid extract of the mucous membrane of the stomach a clot forms after a certain time. The solid portion or *curd* contains the fat held together by insoluble calcium paracaseinate, which is formed from the soluble calcium caseinate of the milk by ferment action. The fluid portion or *whey* contains the lactalbumin, lactose, and inorganic constituents.

The nature of the enzyme responsible for the change has been much discussed. The most probable view is that in the stomach of infants and other sucklings a specific enzyme called *rennin* is present. But it also seems to be true that all enzymes that can hydrolyse casein can cause milk to clot. Thus pepsin, trypsin, erepsin and many of the proteolytic enzymes found in plants will clot milk in the presence of a suitable concentration of calcium and of hydrogen ions. Many workers, notably Pawlow, have urged that the clotting of milk by extracts of the stomach is due to pepsin alone, and that a specific rennetic enzyme does not exist. Such results are probably due to the fact that they only studied extracts of the stomach of dogs and pigs, which do not seem to contain rennin. The results obtained by Bang and Hammersten on the comparison of the various enzymatic activities of extracts of the gastric mucosa of the calf and the pig are taken by the author as conclusive evidence of the existence of two separate enzymes. The results obtained by the author on the relative heat destruction of the clotting powers of the two extracts at a definite hydrogen-ion concentration can be readily repeated, and are most convincing. (See Ex. 256.)

The main differences between the pepsin and rennin as enzymes that can clot milk are :

- (1) Pepsin is more stimulated by increasing the concentration of calcium chloride than is rennin.
- (2) Pepsin is almost completely destroyed by heating for 10 minutes at  $38^{\circ}$  C. at  $P_H = 7.25$ . Rennin only loses a small fraction of its activity.
- (3) Heating for 2 minutes at  $70^{\circ}$  C. at  $P_H = 5$  completely destroys rennin, but has no effect on pepsin. Since the clotting power and ordinary peptic action of such an heated solution is the same as the unheated solution, it must be concluded that the clotting action of pepsin is due to this enzyme, and not to another enzyme associated with pepsin. This is in opposition to the view of Bang, who claims that a special clotting enzyme, which he calls parachymosin, is found in pigs' stomach.

The remarkable fact that pepsin can hydrolyse casein to paracasein at a reaction of about  $P_H = 6.7$ , whilst at this reaction it does not act on any other protein, has not been satisfactorily explained.

The first action of proteolytic enzymes on casein is to hydrolyse it to paracasein. If there is a sufficient concentration of calcium ions and the reaction of the medium is neither too acid nor too alkaline, the paracasein is precipitated as the insoluble calcium paracaseinate. Pepsin in markedly acid solution, erepsin in solutions that are about neutral and trypsin in neutral and faintly alkaline solutions can hydrolyse this further to substances allied to the proteoses and peptones ("caseoses"), and, in the case of erepsin and trypsin, to amino-acids.

Rennin is prepared by extracting the mucous membrane of the fourth stomach of a sucking calf with brine. It can be obtained commercially in the solid or liquid form, being extensively used in the cheese industry, and also in the kitchen for the preparation of "junket." The author

has found that certain of the preparations sold recently contain pepsin rather than rennin, due to the fact that the shortage of calves' stomachs has necessitated the use of pigs'.

253. Measure 5 cc. of fresh milk into a tube and place it in a water bath at  $38^{\circ}$  C. for a few minutes. Add 1 cc. of an active preparation of rennet; mix, replace in the bath, and observe from time to time. Note that the milk sets to a solid mass, so that the tube can be inverted without spilling the contents. Replace the tube in the bath and examine it again after about an hour. Note that the clot has contracted, and that a nearly clear "whey" has been expressed.

254. Repeat the experiment with 1 cc. of a 0.5 solution of commercial pepsin in water. A similar result is obtained.

255. To 10 cc. of milk add 3 cc. of 0.2 N. ammonium oxalate to remove the calcium ions. Mix, and divide into three equal portions, and place these in three tubes, labelled A, B and C.

To A add 1 cc. of N. calcium chloride and 1 cc. of rennin.

To B add 1 cc. of rennin.

To C add 1 cc. of boiled rennin.

Place the three tubes in the water bath for 10 minutes. Note that A clots and that B and C do not.

Boil B (to destroy the rennin) and cool under the tap.

To B and C add 1 cc. of N. calcium chloride. A flocculent precipitate of calcium paracaseinate is produced in B, indicating that the enzyme has acted on the casein in the absence of the calcium salts, but that calcium is necessary for the formation of the precipitate.

NOTE.—If the experiment be repeated with pepsin it will be found that on boiling B and adding  $\text{CaCl}_2$  little or no effect is produced. This is due to the fact that pepsin only seems to act on casein in the presence of calcium salts.

### 256. Distinction between Rennin and Pepsin.

To 10 cc. of 0.5 per cent. pepsin in water add 5 cc. of 0.2 M. acid potassium phosphate, and make the volume up to 100 cc. with distilled water. Titrate 20 cc. of the mixture with 0.1 N. soda to a

reaction of  $P_H = 7.25$ , using the method described on page 277, note 3. Suppose that 1.5 cc. of the soda are required.

To 10 cc. of the diluted enzyme add half this amount of 0.1 N. soda, mix and place the tube in the warm bath for 10 minutes. At the end of the heating period add 0.75 of 0.1 N. HCl, mix and cool under the tap. Label the tube A.

To another 10 cc. of the diluted enzyme add 0.75 cc. of 0.1 N. HCl, and then 0.75 cc. of 0.1 N. soda. Mix, and label the tube B. Determine the relative clotting powers of these two solutions on calcified milk as in Ex. 252. It will be found that A has almost lost its action as compared with B.

Perform a similar experiment with a solution of rennin, adding the phosphate as a buffer, diluting to 100 cc. and determining how much soda is required to bring it to  $P_H = 7.25$ . Label the heated rennin C and the control D. On comparing their clotting powers as before it will be found that heating at  $P_H = 7.25$  has comparatively little effect on rennin as compared with pepsin, the clotting power usually being about half that of the control.

NOTES.—1. In class work it is convenient for the Demonstrator previously to determine the amounts of soda and acid required for 10 cc. of the two enzymes. Otherwise the experiment takes a considerable time.

2. If the reaction be more alkaline than  $P_H = 7.25$  the rennin is rapidly destroyed.

3. The following is the result of a typical experiment, the units being calculated as explained in Ex. 252.

|   | Pepsin. | Rennin. |
|---|---------|---------|
| Control .. .. .                         | 21.2    | 23.1    |
| Heated at $P_H = 7.25$ for 10 mins. . . | 0.03    | 11.4    |

### F. Trypsin.

Trypsin is the proteolytic enzyme formed by the interaction of trypsinogen and enterokinase. Trypsinogen is elaborated in the pancreas, and is found as such in fresh pancreatic juice. Enterokinase is secreted by the small intestine, but is also found in nearly all the tissues of the

body in small amounts. It converts trypsinogen to trypsin, but the mechanism of this change is not fully understood. From the fact that a small amount of the kinase can in time activate a large amount of trypsinogen it is probable that the process is enzymatic, and not merely the union of two substances, each of which alone is inactive. It is remarkable that the rate of activation is at first slow, but increases very rapidly as the process nears completion. This is opposed to the rate of action of other enzymes which is most rapid at the commencement, and which falls off as the concentration of the substrate decreases. No satisfactory explanation of this so-called "autocatalysis" has been offered. Enterokinase acts best in a faintly acid medium, but the optimum  $P_H$  has not been studied. Since the contents of the small intestine are still acid when the pancreatic juice is secreted, it is possible that the conditions of the medium are such as will enable the enterokinase to rapidly activate the trypsinogen.

Trypsin acts best in a slightly alkaline medium, the optimum  $P_H$  being about 8.1. In the absence of proteins, which exert a protective action, it is slowly destroyed by alkalis at body temperature. At room temperature, according to the author's observations, it is most stable at about  $P_H = 5.5$ . It is not readily destroyed in solutions as acid as  $P_H = 1.5$ , though at such a reaction it does not act as an enzyme.

According to the observations of J. Mellanby and Wooley, trypsin is more stable in acid solution than when neutral or alkaline. They claim that in the presence of a small amount of free acid trypsin can be heated to  $100^{\circ} \text{C}$ . for 5 minutes without being completely destroyed, whereas in slightly alkaline solution it is completely destroyed at  $60^{\circ} \text{C}$ . They find that the chlorides of barium and calcium are effective agents in preserving trypsin at body temperature.

Trypsin acts on all soluble and on many insoluble proteins. It finally converts them to a mixture of amino-acids and of relatively simple polypeptides. The hydrolysis

of the protein is not complete even with a very prolonged period of digestion. It is much more complete if the protein has been previously submitted to peptic digestion, indicating the presence of certain groupings in the protein molecule that are attacked by pepsin, and not by trypsin. It must be remembered that in the course of natural digestion in the body the proteins are liable to the attack of three proteolytic enzymes, pepsin, trypsin and erepsin, and that it is possible that complete hydrolysis is most effectively and rapidly attained if the three enzymes are presented in due order.

The course of hydrolysis can be followed by Sørensen's method of formal titration, or by the direct estimation of the amino-acid nitrogen by the gasometric method of D. Van Slyke. As stated above, it is most rapid at the commencement of the reaction, falling off as the hydrolysis reduces the concentration of the substrate. The process is a very complicated one to follow mathematically, owing to the variety of substances simultaneously present which are liable to be attacked by the enzyme, and to the fact that the amount of enzyme is continuously decreasing owing to its instability. For the estimation of trypsin it is preferable to rely on the method given in Ex. 259.

#### Preparation of Trypsin from Pig's Pancreas.

The following method is a modification of that of Mellanby and Wooley. It must be noted that the extract obtained does not contain amylase or lipase, both of which are rapidly destroyed by acids.

Obtain the fresh pancreas of the pig (usually sold as the "internal sweetbread"). Free the glandular tissue from fat as completely as possible, mince finely in a machine and weigh. For every gram of the mince add 3 cc. of 0.5 per cent. hydrochloric acid (by weight). This can be prepared with sufficient accuracy by diluting 13.7 cc. of pure concentrated hydrochloric acid (Sp. Gr. 1.16) to make 1000 cc. with distilled water. Stir the mixture well at intervals for 30 minutes. Then add 6.4 cc. of 5 per cent. soda (or 8 cc. of N. soda) for every 100 cc. of the dilute hydrochloric acid originally used. This gives a reaction of about  $P_H = 4.7$ , which results in a readily filterable mass. Stir thoroughly and filter on a large folded paper. To the filtrate, which is usually quite clear, add 10 per cent. soda to reduce the acidity to about  $P_H = 5.5$ , the optimum reaction for the preservation of trypsin. This can be obtained approximately by cautiously adding the soda until a 2 cc. portion of the mixture gives only a faint reddish tinge with a few drops of methyl red. Then add toluol (10 cc. per litre) as a preservative, shake and store in a stoppered bottle in a cool, dark, cupboard. Should the bottle be



opened, it may be necessary to add a little more toluol and to shake well before returning it to store.

Trypsin can also be obtained by extraction with dilute alcohol. The material left over after the preparation of lipase (see p. 158) should be filtered. The filtrate contains amylopsin and trypsin. The trypsin is more permanent if 1 cc. of pure concentrated hydrochloric acid be added for every litre. If amylopsin is required the acid should not be added.

For the following experiments a 1 in 5 or even 1 in 10 dilution of the above extract can be used.

#### 257. Detection of Trypsin by the use of Calcified Milk.

Measure 5 cc. of the prepared milk (see Ex. 252) into two test-tubes labelled A and B, and place them in a water bath at 37° C. To A add 1 cc. of the solution. Boil a little of the solution and add 1 cc. of the cooled solution to B, using a clean pipette. Mix the contents of the tubes separately, replace them in the bath and observe at intervals. If trypsin is present the milk in A will be precipitated or form a clot. The observation is valueless if the milk in B also clots, as may happen with milk which has "turned sour," or even with fresh milk if the fluid tested is very acid.

NOTE.—A positive result indicates the presence of trypsin, pepsin, rennin, or other proteolytic enzyme. Further tests must be applied (Exs. 248, 256, and 258). But it may be noted that trypsin will cause the clot to disappear gradually, a phenomenon not obtained with the other enzymes.

#### 258. Detection of Trypsin by the use of casein solution.

To 10 cc. of the casein solution (see below) add 2 cc. of the enzyme solution, mix, label the tube C, and place it in the water bath at 37° C. Boil a little of the enzyme solution, cool, and add 1 cc. to 5 cc. of the casein. Label the tube D. At intervals of about 10 minutes transfer about 1 cc. of C to a tube, and add 1 per cent. acetic acid, drop by drop. If trypsin is present, it will be found that after a certain interval, depending on the strength of the ferment, it is not possible to produce a precipitate by the addition of acetic acid. Should this stage be reached, confirm the result by obtaining a precipitate of casein in D by careful acidification.

NOTES.—1. *Casein solution.* Weigh out 1 gram. of Hammersten's Casein (which can be obtained from Casein, Ltd., Battersea, London, S.W.) into a clean, dry beaker. Add about 20 cc. of distilled water and stir. Add 10 cc. of 0.1 N. soda and stir well. Allow to stand for about 30 minutes, and make the volume up to 100 cc. with distilled water.

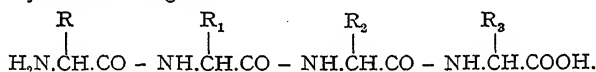
2. Casein is hydrolysed by trypsin in alkaline neutral or faintly acid solution. The first product of hydrolysis is paracasein, which is precipitated

as calcium paracaseinate if there be a sufficient concentration of calcium salts present, as there is in Ex. 257. The later products of hydrolysis are bodies allied to the proteoses (caseoses), peptones, and finally the amino-acids. The failure to obtain a precipitate with acetic acid marks the stage when the last trace of paracasein has been hydrolysed. Though rennin and pepsin in neutral or faintly acid solution can hydrolyse casein to paracasein, they are unable to effect the further stages of break down, and therefore do not give this important test for casein.

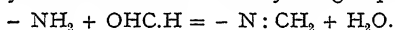
**259. Estimation of Trypsin.** Having demonstrated that a given solution contains trypsin, this can be estimated by following the procedure given in Ex. 252; as first suggested by J. Mellanby. The same unit as that adopted for pepsin is convenient.

**260. The course of tryptic digestion of casein as followed by formol titrations.**

*Principle of the method.* Suppose the constitution of a protein to be represented by the following formula:



The aqueous solution of such a compound would probably be nearly neutral, the acidity due to the terminal  $\text{COOH}$  group being balanced by the basicity due to the terminal  $\text{NH}_2$ . The addition of a small amount of soda would render the solution alkaline to phenol phthalein. On adding a neutralised solution of commercial formaldehyde (formol) the basicity of the  $\text{NH}_2$  group is removed by the formation of a methylene group [see p. 69 (3)].



The whole molecule would now behave as an acid owing to the unopposed influence of the terminal  $\text{COOH}$  group; and the solution would require the addition of an equivalent of soda to make it again alkaline to phenol phthalein. Let this amount of soda be A.

If the protein be hydrolysed by an enzyme into its constituent amino-acids, the amount of soda required for neutralisation would probably be approximately the same as that for the intact protein. But after treatment with formol the amount required to make the solution alkaline to phenol phthalein would be four times greater than A. The reason for this is that after hydrolysis there are four free amino-groups and four free carboxylic groups, and the solution is still about neutral. But after treatment with formol the basic influence of all four amino groups is removed, and to make the solution alkaline enough soda has to be added to neutralise all four carboxylic groups. It therefore follows that the degree of hydrolysis of such a protein can be followed by determining the amount of standard alkali required to neutralise the solution after treatment with formol.

The usual method adopted is to withdraw samples of the digesting mixture at intervals, add neutralised formol and titrate to a definite pink colour to phenol phthalein. The amount required (less the amount for a sample taken immediately after the addition of the enzyme) is a measure of the number of amino-groups set free. In some cases the solution is neutralised to litmus before the addition of the formol. A practical difficulty is that of defining the exact end point of the titration so that the same final reaction is obtained in

the control and in all the observations made. The digestion mixture is usually opalescent and pigmented. A simple control is obtained by boiling a portion of the digest in a flask to destroy the enzyme, adding formol and phenol phthalein and titrating to a definite pink. The control and all subsequent titrations are brought to the same colour. But the best results are obtained by use of the comparator of Cole and Onslow, constructed to hold large boiling tub (see fig. 37). With this it is possible to titrate sharply to a definite  $P_H$ . The principle underlying the method of titration is described in Ex. 322. To save material in this experiment only one buffer solution is used, instead of the two in Ex. 322.

A further point of interest in connexion with formol titrations is the fact that the amount of soda required to make the digestion mixture alkaline to  $P_H = 8.3$  (pink with phenol phthalein) increases during the course of digestion, being especially marked in the early stages of tryptic digestion. This is not observed in the experiment conducted by the method described below, owing to the fact that the formol is added directly to the sample. The relationships between the amounts of alkali required before and after the addition of formol with various proteins subjected to the action of different proteolytic enzymes is being carefully studied, as it seems possible that they will throw light on the nature of the groupings in the protein molecule that are liable to attack by the different enzymes.

*Formol Solution.* Dilute commercial formaldehyde (40 per cent.) with an equal volume of distilled water. Add 10 drops of a 0.5 per cent. solution of phenol phthalein in 50 per cent. alcohol for every 100 cc. of the solution and titrate with 0.1 N. soda until a faint pink tinge is obtained. It may be necessary to add a few more drops of the alkali from time to time owing to the oxidation of the formaldehyde to formic acid.

*Casein Solution.* Make a 10 per cent. solution of commercial casein according to the directions given in Ex. 87 A (i.) to (vi.). Add toluol, shake well, and place in a deep water bath or air incubator at  $38^\circ$  to  $40^\circ$  C. for 24 hours, shaking at intervals. When all preparations for the titrations have been made, there are added 25 to 50 cc. per litre of the pancreatic extract described on p. 212. The bottle is well shaken, a sample immediately withdrawn for titration, and the bottle replaced in the incubator or water bath. Further samples are taken at intervals, the following making a suitable series:  $\frac{1}{4}$ ,  $\frac{1}{2}$ , 1, 2, 6, 24 and 48 hours.

*Method of titration.* Withdraw 20 cc. of the digestion mixture with a pipette and transfer it to the tube (3) of the comparator (fig. 37). Another portion of 20 cc. is placed in tube (2). In (1) place 25 cc. of a buffer solution of  $P_H = 8.5$  (see p. 28). In (4) place about 25 cc. of water. Into tubes (1) and (3) measure 10 drops of 0.5 per cent. phenol phthalein by means of a dropping pipette (fig. 5). To (3) add 5 cc. of the neutralised formol. To (2) add 5 cc. of water to make the colour and opalescence comparable with that of (3). Titrate (3) with 0.2 N. soda from a burette. The end point is reached when the appearance seen on the ground glass screen at Y is the same as that seen at X. If more than 2 cc. of the alkali are required, the same volume of water should be added to tubes (1) and

(2). Thus, suppose that just before the end point is reached 4.5 cc. of the 0.2 N. soda have been added to (3). Add 4.5 cc. of water to (1) and to (2), and then complete the titration till exact equality of tint is obtained.

*Calculation.*

1000 cc. N. soda = 14 grams. of amino-acid nitrogen.

1 cc. of 0.2 N. soda = 2.8 mgm. amino-acid nitrogen.

If (a) = amount of 0.2 N. soda required for 20 cc. of the digestion mixture immediately after the addition of the enzyme, and (b) the amount after an interval of (t) minutes, then  $[(b) - (a)] \times 5 \times 2.8$  = mgms. of amino-acid nitrogen liberated in 100 cc. of the digestion mixture in (t) minutes.

It will be found that the rate of digestion, *i.e.* the mgms. of amino-acid nitrogen liberated in 1 minute, is greatest at the commencement of the digestion, falling off as the concentration of the substrate decreases.

NOTES.—1. If a parallel experiment be conducted with chloroform instead of toluol as the antiseptic, it will be found that the digestion rate is considerably less. For that reason it is preferable to use toluol instead of chloroform for the preservation of trypsin solutions and as an antiseptic in digestions.

2. The percentage amount of the casein digested is much greater in dilute than in strong solution. The addition of an equal volume of water will induce a greater degree of hydrolysis in the above experiment than doubling the amount of enzyme.

## 261. The products of tryptic digestion of casein.

The final products are given on pages 67 to 69. The methods of separation are discussed on p. 70, and the details of the special methods required for the isolation of tryptophane, tyrosine and leucine are given in Exs. 87, 89 and 93 respectively. The following exercise is inserted for the benefit of junior students as illustrating the principles of the methods used.

*Digestion.* The solution and digestion is carried out as described in Ex. 87 A (i.) to (x.), but the digestion period may with advantage be extended to about 2 weeks. The bottle is removed from the incubator, and the contents transferred to a 3 litre flask, and heated on a boiling water bath or in a steamer for about 1 hour. The mixture is then filtered hot and a portion evaporated in the

boiling water bath to about half its bulk. This is distributed into small beakers (labelled B) and allowed to stand in a cool place for 24 hours for the tyrosine to separate out. The remaining portion is distributed into test-tubes (labelled A) and allowed to stand for 24 hours.

(i.) *Bromine reaction for free tryptophane.* If necessary filter A from a crystalline precipitate of tyrosine. Acidify about 5 cc. with a couple of drops of strong acetic acid and add bromine water, drop by drop; a pink colour gradually develops, which deepens and then disappears as more bromine water is added. When the colour is no longer intensified by the addition of bromine, add 2 or 3 cc. of amyl or butyl alcohol and shake. On standing, the alcohol rises to the surface coloured a fine red or violet. (See p. 93.)

(ii.) Treat another 5 cc. of the filtrate with 1 cc. of 25 per cent. sulphuric acid and 10 cc. of a 10 per cent. solution of mercuric sulphate in 5 per cent.  $\text{H}_2\text{SO}_4$ . Shake the tube and leave it for 10 minutes. Note the yellow precipitate of a mercury compound of *tryptophane*. Filter this off and label the filtrate C. Wash the precipitate through a hole in the paper into a clean tube, fill with water, shake and filter again, neglecting the filtrate. Wash the precipitate on the paper once more with water and then let it drain. Scrape a portion off the paper, transfer it to a tube, add 2 cc. of "glyoxylic reagent" and then 2 cc. of concentrated sulphuric acid. A purple colour is produced, showing that tryptophane is responsible for the glyoxylic reaction. (See Ex. 23.)

Treat another portion of the precipitate with Millon's reagent and boil. A yellow colour is produced, not the characteristic red of Millon's reaction.

To another portion of the precipitate apply the xanthoproteic test. A well-marked reaction is obtained. (See Notes to Ex. 21.)

To portions of filtrate C apply the glyoxylic, Millon's and the xanthoproteic reactions. Only the latter two are obtained, the tryptophane, but not the tyrosine, having been removed by the mercury reagent employed. It will be noted that the solution becomes bright pink as soon as the Millon's reagent is added. This is due to the influence of the sulphuric acid. On heating the colour may be discharged if an excess of the reagent be used.

(iii.) Examine the crystalline deposit in A, or, failing this, the mass in B. Under the lower power of the microscope, tyrosine will appear as sheaves or fan-shaped aggregates of needles. The material in B may also contain spheres or cones with a radiating striation consisting of leucine. Should these not be found, the crystalline mass should be filtered off by use of a suction pump and the filtrate concentrated on a boiling water bath. On standing for 24 hours the characteristic leucine balls will separate out.

### G. Erepsin.

This is a proteolytic enzyme widely distributed in the animal and vegetable kingdoms. In animals it is especially abundant in the mucous membrane of the intestine, particularly in the jejunum. It is found in the succus entericus.

It differs from trypsin in that it has no action on such native proteins as albumins and globulins. But it hydrolyses the proteoses and peptones and also casein. The final products of action are the same as in the case of trypsin, that is, free amino-acids. But it would seem that it can break down many of the polypeptides that are resistant to the action of trypsin. Thus the products of ereptic digestion may fail to give the biuret reaction, whereas the most prolonged tryptic digests give a vivid reaction owing to the polypeptides present. The enzyme is probably of great importance in protein digestion in completing the hydrolysis initiated by the successive action of pepsin and trypsin. It is usually stated that the optimum  $P_H$  for the action of erepsin is 7.8.

*Preparation.* Obtain a length of the small intestine of a recently killed pig. Wash out the contents under the tap, split open, and spread on a board with the mucous membrane uppermost. Scrape this off the muscle coats with the back of a scalpel, placing the material in a weighed dish. Determine the weight of mucosa obtained. Grind it with sand, add about 15 times its weight of water and transfer to a flask. Add some toluol and shake. After standing for about half an hour add 1 gram. of sodium chloride for every 100 cc. of water taken, shake till dissolved, and filter through a pleated paper. Filtration is very slow, and may take several hours. It can be accelerated by the addition of 4 cc. of 0.1 N acetic acid for every 100 cc. fluid, which precipitates some of the mucin and nucleo-proteins which make the solution so slimy. It is

perhaps safer to use the untreated, unfiltered suspension of the mucosa. Toluol should be added to the flask in which the filtrate is collected. The various enzymes in the fluid are not very stable, so that the material must be obtained and the extract made not more than two days before it is required.

262. **Action of erepsin on peptone.** Label two 150 cc. flasks A and B, and into each place 100 cc. of a 2 per cent. solution of commercial peptone. To A add 10 cc. of the filtered extract and 2 or 3 cc. of toluol, shake well, and stopper with a cork. Boil another 10 cc. of the extract to destroy the enzymes, cool, and add to B. Add toluol and stopper. Incubate the flasks at 38° to 40° C. for 24 to 48 hours or longer.

(i.) To 5 cc. of each add two or three drops of strong acetic acid and then bromine water, drop by drop (see Ex. 261). A pink colour is obtained in A, but not in B, showing that the tryptophane bound in the peptones has been set free by the action of a ferment.

(ii.) Titrate 20 cc. of A and B according to the directions given in Ex. 260. A considerable increase in amino-acid nitrogen results, owing to the splitting of the peptide linkages of the peptones by the action of erepsin.

(iii.) To 1 cc. of A and B add 4 or 5 cc. of water, 2 drops of 1 per cent. copper sulphate, and 1 or 2 cc. of 5 per cent. soda. A strong biuret reaction (Ex. 24) is given by B, whereas A may give none, or only a feeble reaction.

NOTE.—It is not always possible to obtain a solution that fails to give the biuret test. It seems to depend on the quality of the peptone employed.

263. **Action of erepsin on casein.** Prepare a 2.5 per cent. solution of casein in dilute alkali by diluting 1 part of the solution described in Ex. 87 with 3 parts of water. To 50 cc. add 10 cc. of the intestinal extract and toluol and label A. To another 50 cc. add 10 cc. of water (or of a boiled intestinal extract), toluol, and label B. Incubate the stoppered flasks for 2 to 7 days at 38° to 40°.

To portions of the resulting solutions carefully add dilute acetic acid, drop by drop. A precipitate of unchanged casein is produced in B. In A the precipitate is much less or may be absent.

To the acidified solutions thus obtained add bromine water, drop by drop. A reaction for free tryptophane is produced in A, but not in B.

### H. Amylopsin.

This amylolytic enzyme is secreted by the pancreas. According to most observers it has an action identical with that of ptyalin.

J. Mellanby and Wooley\* state that pancreatic juice alone converts starch to stable dextrin (25 per cent) and maltose (75 per cent.), but that after treatment with small amounts of acid it can carry the hydrolysis as far as glucose, owing to the appearance of maltase, the enzyme which converts maltose to glucose. Since the pancreatic juice on entering the small intestine is mixed with the acid chyme it is probable that the observations quoted are of considerable importance.

Extracts of the pig's pancreas normally contain maltase as well as amylase. The enzymes are destroyed by acids, and so are not found in the extract described on page 212. According to Mellanby and Wooley the amylopsin is best obtained by extracting the minced pancreas with twice its weight of pure glycerol for 24 hours at 37° C.

*Preparation.* The pancreas is extracted with dilute alcohol as described on p. 158. After standing 3 days the mass is filtered. It is not necessary to add a preservative, the alcohol acting as such. The amylase and maltase are somewhat unstable, the best results being obtained with recently prepared extracts.

264. **The action of amylopsin on starch.** To 20 cc. of a 3 per cent. solution of soluble starch add 1 cc. of 5 per cent. sodium chloride, divide into two equal portions, and place them into two test-tubes, labelled A and B. To A add 1 cc. of the pancreatic extract and a few drops of toluol. Shake, stopper, and incubate for 24 hours at 38° to 40° C. To B add about 1 cc. of saliva, then toluol, and incubate with A.

To the digested fluids add phenyl-hydrazine hydrochloride, sodium acetate, and acetic acid, and proceed as directed in Ex. 109. Examine the tubes after they have been in the boiling water bath for 30 minutes. A will probably contain a precipitate of phenyl-glucosazone, whilst B will remain clear (see Ex. 122). Examine a

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\* *Journ. of Physiology*, xlix., p. 246.



portion of the deposit in A under the microscope and note the characteristic crystals of the glucosazone. In B the sugar produced is maltose, the osazone of which does not separate in the boiling water bath.

265. **The action of pancreatic maltase.** Measure 10 cc. of a 2 per cent. solution of maltose into two tubes, C and D. To C add 2 cc. of the pancreatic extract and a little toluol. Boil 2 cc. of the extract, cool and add it to D, together with a little toluol. Stopper the tubes and incubate for 24 to 48 hours at 38° to 40° C. Prepare the osazones as described in the previous exercise. C generally gives a fair yield of glucosazone. In D there is no separation whilst hot, but on cooling slowly the characteristic crystals of maltosazone separate out (Ex. 122).

### I. Maltase, Lactase and Sucrase.

These enzymes hydrolyse the three chief disaccharides into their constituent monosaccharides. They are all found in the succus entericus and the mucous membrane of the small intestine. Maltase is also present in pancreatic extracts (see above).

By the action of these enzymes the sugars of the food and those formed by the action of ptyalin and amylopsin in starch are converted to glucose, fructose and galactose before absorption. The disaccharides themselves are never found in the blood, and if injected they are rapidly excreted in the urine.

266. **Maltase.** To 50 cc. of a 2 per cent. solution of maltose add 10 cc. of the extract of pig's intestine (see p. 218) and a little toluol. Transfer to a small flask labelled E, stopper, shake well, and incubate for 24 to 48 hours (or longer) at 38° C. Boil 10 cc. of the extract and cool under the tap. Add it to another 50 cc. of the maltose, label the flask F, add toluol, and incubate as a control.

With 10 cc. of E and F proceed to prepare the osazones as directed in the previous exercise. E generally gives a good yield of glucosazone, whilst the solution is still hot, whilst F only yields the maltosazone on cooling.

The remaining 50 cc. of the solutions may be treated with 5 cc. of the A mercuric nitrate solution (p. 391) and filtered. On examining the clear filtrates polarimetrically it will be found that the rotation in E is less than in F, owing to the fact that the specific rotatory power of glucose is less than that of maltose.

It is usually possible to demonstrate the presence of glucose in E by the author's method, using blood charcoal. Five cc. of the two solutions are diluted with 15 cc. of water, and the parallel tests conducted as described in Ex. 381.

267. **Lactase.** Repeat the above exercise, using a 2 per cent. solution of lactose instead of the maltose. The osazone method usually gives a definite result, as does the method with blood charcoal. Polarimetric observations are of little use with lactose, owing to the fact that the rotation of the solution is only slightly changed by the hydrolysis of lactose.

268. **Sucrase (Invertase).** To 20 cc. of 2 per cent. sucrose add 5 cc. of the intestinal extract and a little toluol; shake, label, stopper and incubate for 24 to 48 hours. Perform a control experiment by first boiling the extract.

Examine the two solutions for reducing sugar, which appears as the result of the action of the enzyme.

### J. Bacterial decomposition in the intestine.

A large number of micro-organisms are common inhabitants of the intestine, being especially abundant in the colon. Their growth and activities are dependent on a variety of conditions, such as reaction, supply of food materials, etc. It is probable that the changes induced, even by a specific organism, are affected by the action of other bacteria. For example, the fermentation of carbohydrates to lactic and other acids by certain organisms may inhibit the growth or modify the chemical activities of other types.

It is not possible to give here a full account of the various changes brought about by bacteria in the intestine, but certain of the products formed by the putrefaction of

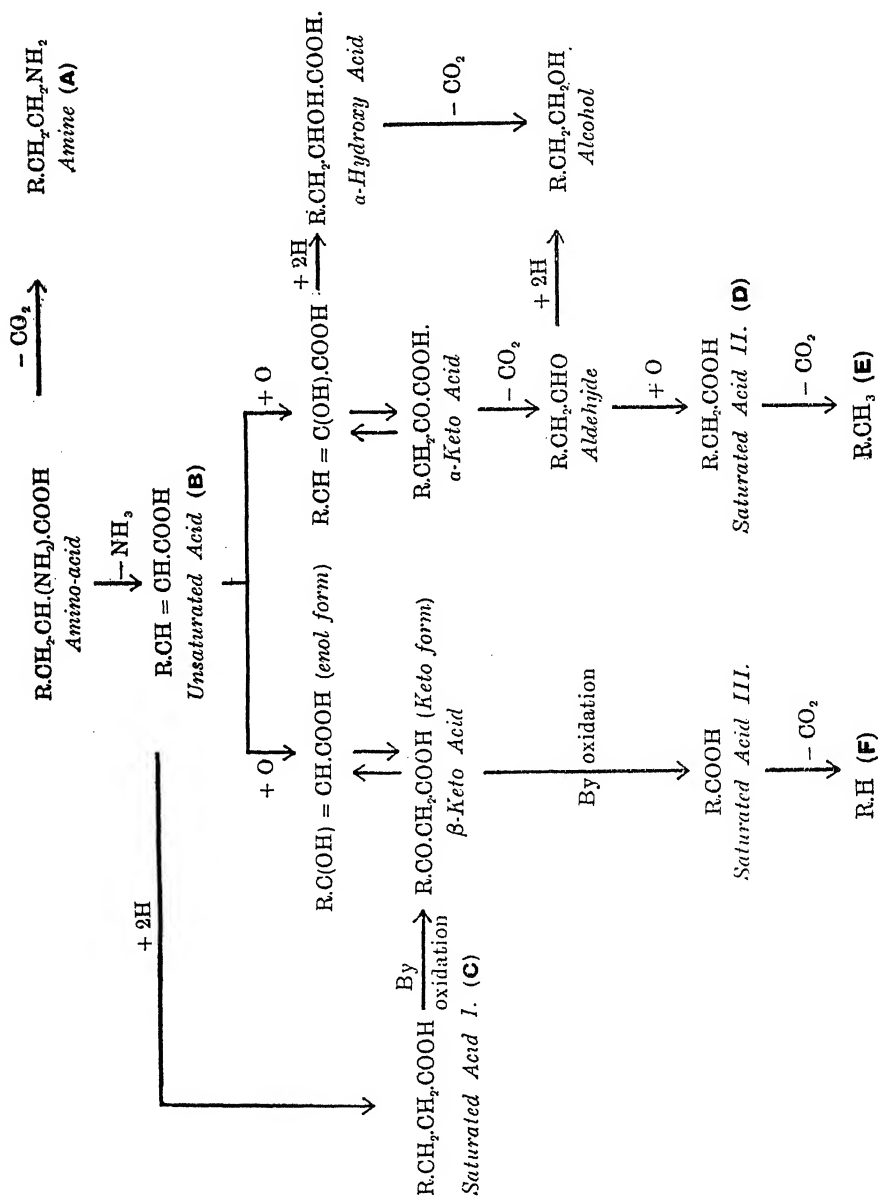
the proteins and amino-acids have such powerful physiological actions that they merit special attention.

It is probable that undigested protein is more liable to putrefaction than are the amino-acids themselves, since the latter are rapidly absorbed from the small intestine, where, normally, bacterial action is not very pronounced. For that reason, if the proteins of the food are well cooked, thoroughly masticated, and readily digested, the ill-effects due to the absorption of the decomposition products are not likely to occur.

The changes are probably due to the action of enzymes secreted by the growing organisms. But such enzymes are not easily extracted from their bodies. The order in which the changes occur has been much discussed. Mr. H. Raistrick has made a special study of certain of the reactions involved, and has suggested the scheme given on the next page as indicating the lines on which the decomposition takes place.

The following list gives examples of the substances corresponding to the types A to F of the scheme, which are formed by the bacterial decomposition of tyrosine, tryptophane and histidine.

|                       | Tyrosine.   | Tryptophane.          | Histidine.                                 |
|-----------------------|---|-----------------------|--|
| A. Amines.            | ( <i>p</i> - oxy - phenyl-ethyl - amine) or Tyramine. | Indol ethyl amine.    | $\beta$ -iminazol-ethylamine or Histamine. |
| B. Unsaturated.       | —   | —                     | Iminazol acrylic acid or Urocanic acid.    |
| C. Saturated Acid I.  | <i>p</i> - oxy - phenyl-propionic acid.               | Indol propionic acid. | Iminazol propionic acid.                   |
| D. Saturated Acid II. | <i>p</i> - oxy - phenyl-acetic acid.                  | Indol acetic acid.    | —  |
| E.                    | <i>p</i> -cresol.                                     | Scatol.               | —  |
| F.                    | Phenol.   | Indol.                | —  |



The amines are of considerable importance. Even the relatively simple compounds mentioned above are very active physiologically, causing contraction of non-stripped muscle, which generally results in a rise of blood pressure. Histamine is a very powerful drug, and causes contraction of the uterus. These bases are present in ergot, and are produced by the action of micro-organisms on the proteins of the infected rye. The physiological activity of ergot is mainly due to the presence of tyramine and histamine.

In addition to the putrefaction bases mentioned above, many others have been identified. These bases are sometimes known as "ptomaines."

*Putrescine*,  $\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$ , is derived from ornithine by the loss of  $\text{CO}_2$ . Ornithine itself is  $\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$ , and is obtained by the hydrolysis of arginine (see p. 69).

*Cadaverine*,  $\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$ , is similarly formed by the decarboxylation of lysine.

A complete account of these compounds is given in Barger's "The Simpler Natural Bases."

Of the other substances mentioned, *urocanic acid* was originally isolated from the urine of a dog; hence its name. Raistrick has recently obtained it from histidine by bacterial decomposition.

*Phenol* and *p-cresol* are mentioned again on p. 282.

*Indol* and *scatol* are mainly responsible for the characteristic faecal odour. They are important to the practical bacteriologist, owing to the fact that only certain organisms are able to produce them from tryptophane. For this reason certain suspected organisms are grown in a suitable medium, which is tested for indol after a given time. The information thus obtained is used in arriving at a diagnosis of the organism. The usual medium used is a solution of Witte's peptone, and the incubation period is 2 to 8 days.

Now peptone does not contain free tryptophane, and some specimens of peptone only contain small quantities of combined tryptophane, as can be determined by the failure to get a vivid glyoxylic reaction (Ex. 23). Before the bacteria can make indol it is probable that they have to multiply considerably so as to produce the various enzymes concerned. If the nutrient medium contains free tryptophane and is otherwise suitable, the formation of indol starts almost immediately. Cole and Onslow\* have described a "tryptic broth," prepared by digesting a solution of commercial casein with trypsin, as being eminently suitable for making indol tests. It contains an abundance of tryptophane and other amino-acids in the free state. The growth of the organisms is usually very luxuriant, and the element of uncertainty about the appearance of indol is abolished. With this medium the production of indol by a typical indol former can be detected in four or five hours.

One of the best known indol formers is *B. coli*, an almost universal inhabitant of the intestine. Organisms closely allied to it, such as *B. typhosus* and *B. paratyphosus* are unable to transform tryptophane into indol or scatol.

It is interesting to note that the simultaneous fermentation of glucose inhibits indol production. This is not due to the fact that the medium becomes acid owing to the carbohydrate fermentation. It indicates that the metabolism and enzyme production of the organisms are profoundly influenced by the available sources of energy. Since a large number of diseases are caused by the products of bacterial action, a complete study of the chemistry of bacterial growth is of great importance.

For the following experiments the appliances and technique of a bacteriological laboratory are required.

*Preparation of "tryptic broth."* A 10 per cent. solution of commercial casein is prepared as described in Ex. 87, A (i.) to (vi.). Toluol is added as an antiseptic, but the sodium fluoride is omitted. Trypsin is then added and the

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\* *The Lancet*, July 1, 1916, p. 9.

digestion carried out as described on p. 88, except that it can proceed for 10 to 16 days if convenient. At the end of the digestion period the mixture is transferred to a flask, treated with 150 cc. of a 1 in 10 dilution of pure concentrated hydrochloric acid, heated in a steamer for 30 to 60 minutes and filtered. The filtrate is treated with 5 per cent. sodium hydroxide until it is *faintly* alkaline to litmus. The resulting fluid or "stock broth" can be preserved in a stoppered bottle for a very considerable period if a little toluol be added. One part of the stock broth is treated with two volumes of a 0.4 per cent. solution of sodium chloride and the reaction adjusted to  $P_H = 7.3$  to  $7.4$  by the method given in Ex. 322, Note 3. The "tryptic broth" thus obtained is distributed into convenient flasks, which are plugged with cotton wool and sterilised in the autoclave.

A flask (labelled A) of the cooled sterile broth is inoculated with a culture of *B. coli* (obtainable from a bacteriological laboratory) under the precautions usually adopted to prevent accidental contamination, incubated for 2 days or longer. For comparison an uninoculated flask (labelled B) of the sterile broth is also incubated.

**269. Ehrlich's test for indol.** To about 3 cc. of A and of B add an equal volume of Ehrlich's reagent (see p. 390). A fine red colour appears in A, but not in B.

**NOTE.**—If the test does not succeed it is usual to add 3 cc. of a saturated solution of potassium persulphate. The author has never been able to recognise the advantage of this in improving the delicacy of the test.

**270. Nelson's vanillin test for indol.** To about 3 cc. of A and B add about 5 drops of a 5 per cent. solution of vanillin in alcohol and then about 3 cc. of pure concentrated hydrochloric acid. An orange colour is produced in A, due to the presence of indol or scatol. In B a slight purplish tint may develop on standing, due to a reaction with unchanged tryptophane.

**271. Destruction of tryptophane by *B. coli*.** To 5 cc. of A and B add a drop or two of acetic acid and then bromine water, drop by drop, until no further increase in the colour is obtained. Warm slightly, add 5 cc. of butyl or amyl alcohol to each, shake and allow to stand. The alcohol layer is more deeply coloured in B than in A, owing to the conversion of the tryptophane to indol and scatol.

**272. Tryptophane is the only mother-substance of the indol bodies.** 100 grams. of commercial casein are hydrolysed by boiling with 500 cc. of 1 in 4 sulphuric acid for 12 to 16 hours under a reflux condenser. The resulting dark purple solution is diluted with water.

and the sulphuric acid removed by the addition of a hot saturated solution of baryta, which should be added until the reaction is faintly alkaline to litmus paper. The barium sulphate is filtered off by means of a Buchner funnel. The filtrate is cautiously treated with dilute sulphuric acid until no further precipitate is obtained and filtered again. The filtrate is made faintly alkaline to litmus, and diluted, if necessary, to make a total volume of about 4 litres. 0.5 gram. per cent. of sodium chloride is added, and the reaction adjusted to about  $P_H = 7.35$ . It is then distributed into flasks and sterilised in the autoclave. One flask, labelled C, is inoculated with a culture of *B. coli* and labelled C. Another flask is treated with 0.1 gram. per cent. of pure tryptophane, heated for ten minutes on a boiling water bath, cooled, inoculated with *B. coli*, and labelled D. Another flask, labelled E, is taken as a control. The three flasks are incubated for 2 to 5 days at  $38^\circ$  to  $40^\circ$  C.

(i.) On portions of the three fluids perform tests for indol by Exs. 269 and 270. They are only obtained in D.

(ii.) Apply the glyoxylic test or the bromine test (Ex. 88, A and E) for tryptophane to E. Neither are obtained, owing to the destruction of tryptophane during the acid hydrolysis of the casein. The experiment proves that tryptophane is the only amino-acid which yields indol on bacterial decomposition.

### K. Autolysis.

When an organ is removed from the body and incubated for several days at  $38^\circ$  C. in the presence of an antiseptic such as toluol, it is found that a certain amount of the tissue proteins have been hydrolysed to amino-acids. This disintegration of the tissues is known as "autolysis." It is due to the action of certain proteolytic enzymes which seem to be present in nearly all tissues, and which are sometimes known as "tissue erepsins." But it must be noted that intestinal erepsin does not act on the native proteins, but only on proteoses, peptones and polypeptides. There must be an essential difference between the two enzymes.



The rate and degree of hydrolysis varies considerably with the nutritive condition of the tissue at the moment of death and on the reaction of the medium in which the autolysis proceeds. In general it may be stated that it is accelerated by the previous starvation of the animal and by an acid reaction, the optimum being between  $P_H = 5$  and  $P_H = 6$ .

The fact that autolysis is accelerated by starvation suggests that it is the mechanism by means of which the amino-acids liberated are deaminised to ammonia and an acid. The latter is oxidised to  $CO_2$  and water, either directly or after passing through the stage of carbohydrate. These products being removed by the lungs, the net result is a supply of ammonia for the neutralisation of the acid originally causing the disturbance. It is noteworthy that the products of autolysis contain much more ammonia than is found in the products of action of trypsin and erepsin.

273. **The autolysis of ox kidney.** Ox kidney is freed from fat, finely minced in a machine and weighed. The pulp is treated with 3 times its weight of 0.2 per cent. acetic acid and shaken with toluol, as a preservative. The mixture is incubated for 3 or 4 days at  $38^\circ C$ . It is then filtered from an insoluble residue of haematin, nuclein, etc. The filtrate is treated with more toluol and incubated for another 7 to 9 days.

A. Boil about 10 cc. Note the heat coagulation of undigested native protein. It may be necessary to adjust the reaction to get complete coagulation. Filter off the coagulum and to a portion of the filtrate add bromine water, drop by drop. A red or purple colour indicates the presence of *free* tryptophane (see Ex. 261).

B. Boil another portion of the mixture as before. Cool 10 cc. of the filtrate, add 10 drops of strong sulphuric acid and cool again. Add an equal volume of the mercuric sulphate reagent of Hopkins and Cole (see p. 89), mix and allow to stand for 10 to 20 minutes. A precipitate is obtained which consists of mercury compounds of tryptophane and of the purine bases. Filter.

|   |   |   |
|---|---|---|
| <p><i>Precipitate.</i> On small portions try the glyoxylic reaction for tryptophane and Millon's reaction for tyrosine. The former is intense: the latter negative or feeble.</p> | <p><i>Filtrate.</i> On portions apply the same two reactions. Millon's is intense: the glyoxylic is negative or feeble. To the remainder add 40 per cent. soda drop by drop until the reaction is nearly neutral. Filter.</p> |   |
|   | <p><i>Precipitate.</i> Apply Millon's test. A fairly strong reaction is obtained.</p>   | <p><i>Filtrate.</i> Apply Millon's test. A negative or feeble reaction is obtained.</p> |

Tryptophane is precipitated by the mercuric reagent, even in the presence of 7 per cent. sulphuric acid. Tyrosine is not precipitated in this strength of acid, but is precipitated in a solution that is only faintly acid.

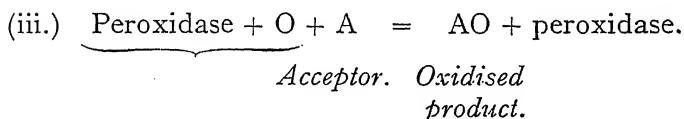
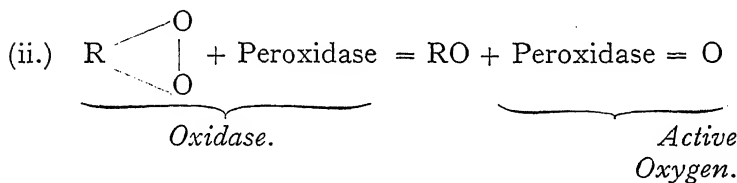
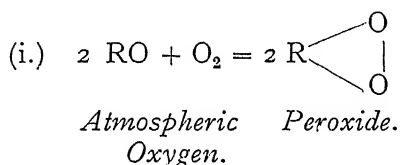
It is not easy to obtain crystals of tyrosine or leucine by direct evaporation of the original solution. This is apparently due to the presence of purine bases and other compounds. If these be first removed by precipitation with phosphotungstic acid in 3 per cent. sulphuric acid solution (the fluid also being acidified to this extent) and the two acids be removed by baryta, the filtrate gives crystals of tyrosine and leucine on evaporation.

#### L. Oxidases, Peroxidases, and Tyrosinase.

The mechanisms concerned in the oxidations in the body of such relatively stable substances as fats and carbohydrates are by no means fully understood. It is generally believed that it is due to the action of enzymes, which are either very unstable or are only active when associated with living protoplasm. This would account for the fact that the ingestion of protoplasmic poisons, such as quinine, has a distinct inhibitory influence on various oxidative processes in the body. There is an increasing amount of evidence to indicate that the sulphur con-

stituents of the cell are intimately related to oxidative processes.

Extracts of various plant tissues possess the property of inducing the oxidation of certain aromatic substances, either directly or after the addition of hydrogen peroxide. The usual nomenclature is that an *oxidase* acts directly, whilst a *peroxidase* requires the addition of hydrogen peroxide. One view as to the mechanisms concerned is that the complete oxidase system consists of a peroxidase and an organic peroxide. The peroxidase deprives the peroxide of an atom of oxygen and transfers it to the substance that is oxidised (the *acceptor*). This view can be represented as follows :



On this view the expressed plant juices or extracts which give the direct oxidase reaction contain an organic substance (RO) which can form a peroxide. When this organic substance is absent, hydrogen peroxide must be added to complete the oxidase system.

The view outlined above is based on the results obtained when the acceptor is guiaconic acid. This is present in tincture of guaiacum resin and is converted to guaiacum

blue by oxidation. Various other aromatic substances, such as benzidine,  $\alpha$ -naphthol and para-phenylene-diamine can be used as acceptors, but in such cases it is found that hydrogen peroxide must usually be added, even to an extract which behaves as a complete oxidase system towards guaiacum. Some very interesting results have recently been obtained by Mrs. Muriel Wheldale Onslow, relating the establishment of the oxidase system in certain plants to the browning that takes place on injury and to the preliminary oxidation of some aromatic cell constituent. They will shortly be published in the *Biochemical Journal*.

The tyrosinases are oxidising enzymes which act on tyrosine. They also act on substances of allied constitution, such as *p*-cresol. The relationships of these plant enzymes to the mechanism of the oxidations occurring in the animal body is not at present certain.

*Preparation of guaiacum tincture.* Take the inner portions of a lump of resin and make a 1.5 per cent. solution in 95 per cent. alcohol, by heating in a flask in a boiling water bath. Add some adsorbent charcoal, boil for about 5 minutes and filter. The solution should be freshly prepared.

A more reliable preparation of guaiaconic acid is described by Lyle and Curtman (*Journ. Biol. Chem.*, xxxiii., p. 1).

274. **Potato oxidase.** Thoroughly wash and scrub a potato. Peel it and pound the peel in a mortar with a little cold water. Filter. Treat a small amount of the filtrate with a few drops of the tincture of guaiacum. A blue colour is obtained after a short time. Boil a few cc. of the aqueous extract, cool and add guaiacum. No blue colour is obtained, showing that the enzyme is destroyed by boiling.

275. **Conversion of Potato oxidase to a peroxidase.** Heat about 5 cc. of the potato extract to 65° C. for 10 minutes in a water bath. Divide the solution into two portions, A and B. To A add a few drops of guaiacum tincture and a few drops of hydrogen peroxide. To B add a few drops of guaiacum tincture. A generally goes blue, whilst B generally gives a negative or very feeble reaction.

NOTE.—The organic peroxide is more unstable to heat than the peroxidase. After heating, the solution requires the addition of hydrogen peroxide.

276. **Horseradish peroxidase.** Horseradish scrapings are soaked in alcohol, filtered and dried in a thin layer. The dried scrapings are extracted with water and filtered. Place 2 or 3 cc. in labelled test-tubes and try the following experiments, a few drops of guiacum tincture or of 10 volumes hydrogen peroxide where indicated.

- A. Extract + guiacum.
- B. Extract + guiacum +  $\text{H}_2\text{O}_2$ .
- C. Guiacum +  $\text{H}_2\text{O}_2$ .
- D. Boiled extract + guiacum +  $\text{H}_2\text{O}_2$ .

If satisfactory reagents are employed a blue colour is only produced in B.

277. **Potato tyrosinase.** Prepare an extract of potato peel as described in Ex. 274. Boil a little tyrosine with about 5 cc. of distilled water and cool to about  $40^\circ\text{C}$ . Make the following mixtures:—

- A. Extract + tyrosine suspension.
- B. Extract alone.
- C. Boiled extract + tyrosine suspension.

Incubate at  $37^\circ\text{C}$ . and note the appearance at intervals. Both A and B darken, but A much more than B.

## CHAPTER IX.

### THE COAGULATION OF BLOOD.

In spite of the immense amount of work that has been done on the subject, it is impossible to explain fully the fact that when blood is shed it rapidly sets to a jelly, which subsequently contracts. The *clot* consists of the corpuscles entangled in a contracting meshwork of fibrin : the yellowish fluid that exudes is the *serum*.

The following account of some of the factors concerned and the appended scheme of their interaction does not allow for a great deal of important work that has been done in recent years. It is almost certain that the phenomenon is considerably more complicated than that indicated below.

#### Factors concerned.

1. *Fibrinogen*, a globulin, present in blood-plasma. It is soluble in dilute salt solutions, acids and alkalies, insoluble in distilled water. It coagulates at 57° C. It is precipitated by half-saturation with sodium chloride.

2. *Pro-thrombin*, a substance of unknown composition, found in plasma, attached to the fibrinogen. It is destroyed by boiling.

3. *Thrombokinase*, a substance found in all tissues and also liberated in the blood by the disintegration of leucocytes and blood-platelets. It converts pro-thrombin into thrombin, under certain conditions.

4. *Calcium salts*, found in plasma, and necessary for the action of thrombokinase. The calcium salts must be of such a nature that they are ionised in solution.

5. *Thrombin*, a ferment formed by the interaction of 2, 3 and 4. It probably splits fibrinogen into serum-globulin and fibrin. The latter, being insoluble in the constituents of normal plasma, comes out of solution and with the corpuscles forms the clot.

The scheme on the following page represents the interaction of the above factors.

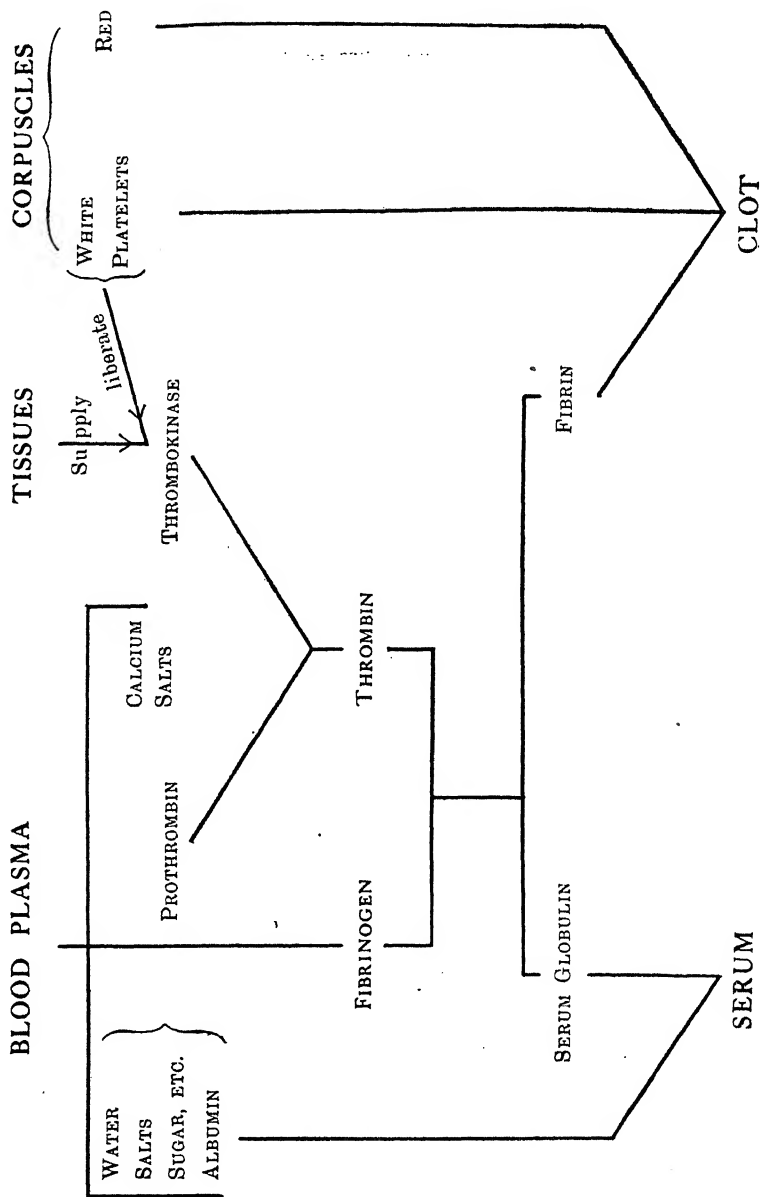
Coagulation is hindered by

1. Cooling.
2. Substances which precipitate calcium salts, or convert the calcium into the non-ionised condition, as oxalates, citrates and soap solutions.
3. Alkalies, which prevent the liberation of thrombokinase by the corpuscles, delay the action of thrombin, and tend to dissolve fibrin.
4. Strong salt solutions, which have a similar action.
5. Anti-thrombin, a substance found in small amounts in the plasma, and in relatively large amounts in extracts of the head of the leech. It combines with thrombin to render it inactive.
6. Anti-kinase, found in the blood, after the slow injection into the blood stream of certain substances, as tissue-extracts, certain snake-venoms, etc.
7. Fluorides, which precipitate calcium salts and prevent the liberation of thrombokinase.

**Preparation of fibrin ferment (thrombin).** Blood serum is treated with four or five times its volume of strong alcohol, well stirred and allowed to stand for two or three days. The precipitate is collected, dried on filter paper in the air, and extracted with water. The filtered extract contains fibrin ferment.

**Preparation of "salted" plasma.** Two litres of water are placed in a large bottle or jar (provided with a tightly-fitting stopper) and the level of the fluid marked by a label. The water is poured off and 400 cc. of a saturated solution of magnesium sulphate substituted. Blood is collected in the bottle till the level is reached, care being taken to ensure thorough mixing with the salt solution by stopping the flow of blood from time to time and turning the bottle upside down. The corpuscles are removed by centrifugalisation and the plasma pipetted off. It should be kept in a refrigerator till required.

278. **The clotting of salted plasma.** Take 2 cc. of salted plasma in a test-tube, add 10 cc. of water, and divide into two portions, A and B. To A add a few drops of fibrin ferment (or of





serum). Place both tubes in the warm bath at 40° C. and examine from time to time. Clotting takes place in both tubes, but much more rapidly in A than in B.

NOTE.—Dilution with water decreases the concentration of the magnesium sulphate, so that any fibrin formed by the ferment (which can now act on the fibrinogen) becomes insoluble in this low concentration of salt.

279. **The preparation of fibrinogen.** To 20 cc. of the salted plasma add an equal volume of a saturated solution of sodium chloride. A precipitate of fibrinogen is formed. Allow the tube to stand for a few minutes and then filter through a small paper. Scrape the precipitate off the paper and treat it with about 5 cc. of 5 per cent. sodium chloride. The fibrinogen dissolves.

NOTE.—If bird's blood be drawn directly into a clean vessel in such a way that contact with the tissues is absolutely avoided, it clots very slowly. This is because the leucocytes are very stable and do not liberate thrombokinase. If this blood be centrifugalised at once, a non-clotting plasma is obtained. Fibrinogen can readily be prepared from this by the method given in Ex. 33. The suspension so obtained is dissolved in dilute salt solution.

280. Divide the solution thus obtained into two portions, C and D. To C add two drops of fibrin ferment. Place both tubes in the warm bath and observe them at intervals. C clots rapidly; D very slowly.

**Preparation of oxalate plasma.** Blood is drawn as in the preparation of salted plasma into a bottle which has 200 cc. of a 1 per cent. solution of potassium oxalate in place of the 400 cc. of saturated magnesium sulphate. The plasma is separated, as before, by centrifugalisation.

281. **The clotting of oxalate plasma.** Dilute 5 cc. of the plasma with 10 cc. of distilled water and divide into three portions, E, F, and G. To E add a few drops of 1 per cent. calcium chloride; to F a few drops of fibrin ferment or serum. Place the three tubes on the water bath and observe them at intervals. E clots in a few minutes; F clots slowly; G does not clot.

**Preparation of fluoride plasma.** This is prepared as oxalate plasma, using a 3 per cent. solution of sodium fluoride in place of the 1 per cent. potassium oxalate.

282. **The clotting of fluoride plasma.** Dilute 5 cc. with 10 cc. of water and divide into three portions, H, K, and L. To H add a few drops of 1 per cent. calcium chloride; to K a few drops of fibrin ferment. Place the three tubes in the warm bath and observe them at intervals. K clots rapidly; H and L do not clot.

## CHAPTER X.

### THE RED BLOOD CORPUSCLES AND THE BLOOD PIGMENTS.

#### A. The Laking of Blood.

The red corpuscles consist of an envelope and meshwork called the stroma, which encloses a solution of haemoglobin and various salts. The stroma consists of a protein, probably a histone, with which is associated a lipoid material, related to cholesterolin and lecithin. The envelope behaves as a semi-permeable membrane to a great many solutions, readily allowing water to pass into or from the corpuscle, but preventing the passage of most salts and other dissolved substances. Thus, if the corpuscles are placed in a solution which has a higher osmotic pressure than the fluid within the corpuscles, water passes out of the corpuscle, which therefore shrinks. Such fluids are called "hypertonic." If they be placed in fluids of a lower osmotic pressure ("hypotonic"), water passes into the corpuscle to equalise the pressures, but salts cannot pass out. The corpuscles swell and the expansion may be sufficient to lead to the disruption of the envelope, so that the enclosed haemoglobin passes into the body of the solution. This bursting of the corpuscles is known as *laking* or *haemolysis*. A solution of the same osmotic pressure as that of the fluid within the corpuscle is said to be "isotonic" or "normal." For mammalian blood 0.9 per cent. sodium chloride is normal; for frog's blood, 0.65 per cent. Other physical means of inducing haemolysis are by repeatedly freezing and thawing the blood, or by warming to 60°C.

The envelopes can also be ruptured by chemical means. Certain substances, such as the bile salts, ether, chloroform,

acids, alkalies, soaps and "saponins" cause the blood to be laked. Some of these act by dissolving the lipoids of the envelope and stroma; others possibly act on the proteins.

If the washed corpuscles be suspended in normal saline containing various buffer solutions, it will be found that haemolysis takes place in all solutions acid to  $P_H = 5.1$ .

Lecithin and cholesterol seem to be somewhat antagonistic in respect to haemolysis, the former accelerating and the latter inhibiting the phenomenon.

Certain pathogenic organisms produce specific haemolysins, notably the tetanus bacillus. The poisonous action of some snake venoms is in part due to the rapid destruction of the red corpuscles.

Haemolysis is brought about by the absorption into the system of a large number of chemical substances, *e.g.* arsine, pyrogallol, toluylenediamine.

Another method of inducing haemolysis is by the addition of certain organic substances developed in certain animals. Thus rabbit's corpuscles that have been washed with isotonic saline are laked when treated with the blood serum of a dog. This haemolytic power of dog's serum on rabbit's blood is very much increased by previously injecting the dog with rabbit's blood.

283. Have two burettes, one containing 1 per cent. sodium chloride, the other distilled water.

Label a series of clean, dry test-tubes A, B, C, etc.

In A place 4.5 cc. NaCl and 5.5 cc.  $H_2O = .45\%$  NaCl.

|   |   |     |   |     |   |         |
|---|---|-----|---|-----|---|---------|
| B | „ | 5   | „ | 5   | „ | = .5 „  |
| C | „ | 5.5 | „ | 4.5 | „ | = .55 „ |
| D | „ | 6   | „ | 4   | „ | = .6 „  |
| E | „ | 6.5 | „ | 3.5 | „ | = .65 „ |
| F | „ | 7   | „ | 3   | „ | = .7 „  |

To each tube add three drops of fresh defibrinated blood,

mix by inverting and allow the tubes to stand for a few minutes. A will be translucent, the corpuscles being fully laked. F will be opaque. Note the dilution which just causes laking. It is generally about 0.55 per cent.

NOTE.—The solution that just causes laking is hypotonic to the blood, indicating that the corpuscles can absorb a considerable quantity of fluid before the envelope is ruptured.

284. To 5 cc. of 0.9 per cent. sodium chloride add some ether and shake vigorously. Then add three drops of blood, mix by inversion. Warm gently and add a few more drops of ether. The blood is laked.

NOTE.—It is essential that pure ether be used. Should the ether be contaminated with acid the blood is precipitated and the pigment converted to acid haematin.

285. To a 0.2 per cent. solution of bile salts in normal saline add three drops of blood. Mix and warm to 37° C. The blood is generally laked, though the experiment does not always succeed.

286. Add some blood to a 2 per cent. solution of urea in water. The blood is laked.

287. Repeat the experiment with a 2 per cent. solution of urea in normal saline. The blood is not laked.

NOTE.—A solution of urea behaves like water as regards the corpuscles. No matter what concentration is used the corpuscles take up water from the surrounding fluid. In other words, the envelope of the corpuscle does not act as a semipermeable membrane as regards urea.

## B. Haemoglobin and its Derivatives.

Haemoglobin (Hb) is a compound protein, being a member of the group of chromoproteins. It is formed by the union of a pigmented non-protein substance containing iron, and called haematin (Hn), with globin, a member of the histone group of proteins.

It is soluble in water and dilute salt solutions: insoluble in ether and alcohol.

It is decomposed by acids and alkalies into haematin and globin. It is decomposed and coagulated by heat.

It forms compounds with oxygen and carbon monoxide, called oxyhaemoglobin ( $\text{Hb-O}_2$ ) and carboxyhaemoglobin ( $\text{Hb-CO}$ ). Both are dissociated into Hb and the gas by exposure to a vacuum.  $\text{Hb-CO}$  is much more stable than  $\text{Hb-O}_2$ , and the avidity of Hb for CO is more than 130 times greater than the avidity of Hb for  $\text{O}_2$ . A small percentage of CO in the air breathed will thus result in the formation of relatively considerable amounts of  $\text{Hb-CO}$  in the blood. This can be converted into  $\text{Hb-O}_2$  by exposure to a high tension of  $\text{O}_2$ , such as is obtained by breathing pure  $\text{O}_2$ .

The  $\text{Hb-O}_2$  obtained from certain animals crystallises readily, but the crystals differ somewhat, according to the animal from which they are obtained. Also the volume of  $\text{O}_2$  combining with 1 gram. of Hb varies, the figure for the horse being 1.34 cc. of  $\text{O}_2$  per gram. of Hb. The oxygen is probably united to the iron of the haematin molecule, the reaction  $\text{Fe} + \text{O}_2 \rightleftharpoons \text{FeO}_2$  being the basis of the reaction  $\text{Hb} + \text{O}_2 \rightleftharpoons \text{Hb-O}_2$ .

The ratio  $\frac{\text{volume of } \text{O}_2 \text{ evolved in cc.}}{\text{weight of iron in grams.}}$  is called the specific oxygen capacity.

Theoretically it is

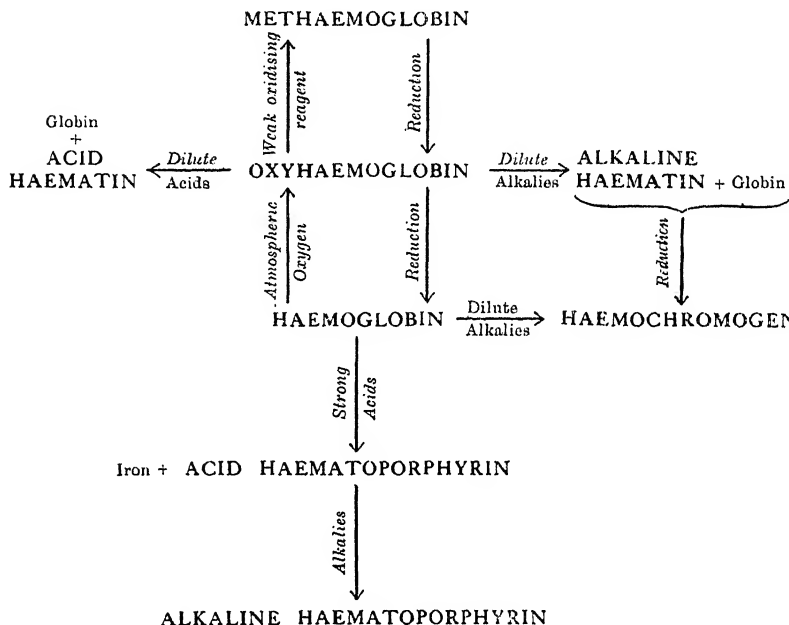
$$\frac{\text{O}_2}{\text{Fe}} = \frac{1 \text{ molecular volume } \text{O}_2}{1 \text{ gram. molecule Fe}} = \frac{22,394}{55.85} = 401.$$

Recent analyses of the blood of various animals have given the value 401.8 which agrees very closely with the theoretical.

The volume of oxygen loosely held by 1 gram. of  $\text{Hb-O}_2$  is 1.345 cc.

So the minimum molecular weight of oxyhaemoglobin is  $\frac{22,394}{1.345} = 16,712$ .

The method of formation of certain of the derivatives of haemoglobin can be represented as follows:—



289. **Crystallisation of oxyhaemoglobin (Rapid method).** To a few cc. of defibrinated dog's blood in a test-tube add ether, drop by drop, till the blood is completely laked. Add to the blood a pinch of finely powdered ammonium oxalate; allow the salt to dissolve by gentle shaking, and let the tube stand. Crystals of oxyhaemoglobin separate out, especially if the solution is cooled to 0° C. by means of ice. Examine them microscopically, and note that they are in the form of thin rhombic prisms.

Make a drawing of the crystals.

NOTE.—This experiment does not always succeed as described. If the blood fails to crystallise out in an hour, place a drop on a slide, spread it out to form a thin layer and leave it for five minutes; cover with a slip and note the crystals of oxyhaemoglobin that form at the edges.

### C. The Spectroscopic Examinations of the Blood Pigments.

#### The use of the Direct-vision Spectroscope.

The instrument described is the small pocket spectroscope, with wave-length scale attached, manufactured by Zeiss and Co. It is to be hoped that an equally good instrument of home manufacture will soon be forthcoming. The instrument (fig. 30) consists of two tubes. The shorter tube A contains a transparent photographic scale of wave-lengths, with a mirror to project its image into the field of vision. By means of the tube D this scale can be focussed, and by the screw F it can be adjusted to its proper position. The tube G contains a series of alternating prisms of crown and flint glass, arranged to allow the spectrum to be observed by the eye in the line of the tube. The tube B which slides on G has a vertical slit, the width of which can be adjusted by turning the collar E.

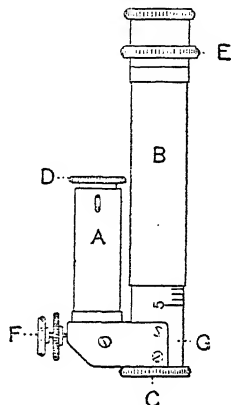


Fig. 30.—Zeiss' direct-vision spectroscope with wave-length scale ( $\times \frac{1}{2}$ ).

*To adjust the spectroscope:* see that D and B are pushed in as far as they will go. Look through C towards the light with A to your left. Cautiously turn E till the spectrum is just visible. (It is most important to use an extremely narrow slit.) Slide B out very slowly (in most instruments for  $3\frac{1}{2}$  divisions as marked on the barrel C) till fine black vertical lines can be seen in the spectrum, and notice particularly a fine black line immediately to the left of the narrow strip of yellow. This line is known as the D line of Fraunhofer. The wave-length of it is  $.59\mu$ , a position indicated on the scale by the division marking it (the one to the right of 0.6) being produced further down than any other. If necessary alter the position of the scale by turning the screw F until the D line exactly coincides with the division mentioned. If the instrument has to be adjusted at night-time, when the D line cannot be observed, set the scale by use of the emission-spectrum of sodium (obtained by placing a few crystals of common salt on the wick of a spirit lamp). The emission spectrum of sodium exactly corresponds to the D line. The scale is so drawn that, if it be set in position as described, the wave-length of light in any part of the visible spectrum can be read directly.

The numbers on the scale indicate wave-lengths in thousandths of a millimetre, the unit being  $1\mu$ . In the more recent patterns the wave-lengths are given in millionths of a millimetre, the unit being  $1\lambda$ . Thus the wave length of the D line is  $589\lambda$ . The other Fraunhofer lines that can be readily observed with the instrument are C ( $657\lambda$ ), E ( $527\lambda$ ), b ( $518\lambda$ ) and F ( $486\lambda$ ).

*To observe absorption spectra:* slightly open the slit of the spectroscope, thus obtaining a better illumination. Direct the instrument to the light, and place the test-tube containing the fluid to be examined directly in front of, and touching, the tube B, with its axis parallel to the slit, taking care not to interfere with the illumination of the scale. With strong solutions of certain pigments observed in this way it is often difficult to avoid illuminating the two ends of the spectrum, the light being reflected from the sides of the tubes, and not passing through the solution. To obviate this it is perhaps better to place the solution in a beaker, remembering that the absorption of light increases

with the depth of layer examined, as well as with the concentration of the pigment. For accurate work the haematoscope should be employed. This is a vessel with parallel glass slides 1 cm. apart.

In handling the instrument the screw F is very liable to be turned, and so the position of the scale to be shifted. From time to time, therefore, the slit should be narrowed, and an observation made to ascertain whether any shifting of the scale in reference to the D line has occurred.

Record the absorption of light of the various pigment solutions on the blank scale, to be found on page 372. Fill in with black pencil marks the exact parts of the spectrum where light is absorbed, leaving the remainder blank. It will not be found advisable to use coloured pencils.

290. **Oxyhaemoglobin.** Take 5 cc. of distilled water in a test-tube, and add one drop of defibrinated blood, shake well and observe the spectrum of **dilute** oxyhaemoglobin. There are two absorption bands in the green. The one near the D line (the  $\alpha$  band) is somewhat narrower and darker than the  $\beta$  band. The middle of  $\alpha$  is about  $\lambda$  578, and that of  $\beta$  about  $\lambda$  540.

291. Add two more drops of defibrinated blood and examine again. The spectrum has become very much cut off, especially at the blue end: the absorption bands have probably merged into one, leaving a little patch of blue light and a broader belt of red light on the two sides. If this effect has not been produced, add a little more blood or dilute with water. Record the spectrum of the solution on the chart as that of a **medium** solution of oxyhaemoglobin.

292. Add another drop or two of defibrinated blood, and note that the blue light becomes absorbed, light only coming through in the red. (**Strong** solution.) If the concentration is still further increased, the red also is absorbed.

NOTE.—It is important to observe that a *medium* solution of oxyhaemoglobin has a single band in the green.

293. **Haemoglobin (reduced haemoglobin).** Treat 5 cc. of water with one drop of defibrinated blood and thus obtain a solution of oxyhaemoglobin of such a strength that it shows two well-marked absorption bands. Add two or three drops of a solution of ammonium sulphide, mix and *warm to about 50° C.*, avoiding any unnecessary shaking. If freshly prepared Stokes' fluid is obtainable, add three or four drops, in which case there is no necessity to warm. Note, in the latter case, that the bright scarlet colour of oxyhaemoglobin gives place to the less vivid colour of reduced



haemoglobin. Examine the solution spectroscopically. There is a *single* broad band in the green which overlaps the space enclosed by the two bands of oxyhaemoglobin, and is fainter than either. Its centre is about  $\lambda$  565.

NOTE.—Stokes' fluid is prepared as follows: dissolve 3 grams. of ferrous sulphate in cold water: add a cold aqueous solution of 2 grams. of tartaric acid and make the solution up to 100 cc. with water. Immediately before use add strong ammonia until the precipitate first produced is redissolved. It rapidly absorbs atmospheric oxygen and must, therefore, be freshly prepared. Its great advantage over ammonium sulphide is that it can be used in the cold, whilst with the sulphide the solution must be warmed.

294. Place your thumb over the top of the test-tube containing the reduced haemoglobin and shake vigorously. Examine immediately with the spectroscope, and note that the two bands of oxyhaemoglobin have reappeared owing to the oxidation of the haemoglobin by the oxygen of the air. If the tube be allowed to stand for a short while, reduction may occur again from excess of reducing reagent present.

295. **Carboxyhaemoglobin.** Obtain some CO-haemoglobin that has been prepared by passing a stream of carbon monoxide or coal-gas through a solution of oxyhaemoglobin. Notice the peculiar bluish tinge of the solution. Examine a portion spectroscopically, and, if necessary, add water till two well-marked bands are visible. Note that they are very similar to the two bands of oxyhaemoglobin. Accurate observation, however, will show that they are both slightly nearer the violet end of the spectrum, the middle of  $\alpha$  being  $\lambda$  572 and of  $\beta$   $\lambda$  535.

NOTES.—1. A small amount of caprylic alcohol added to the blood facilitates the preparation of Hb-CO in preventing excessive frothing.

2. If the student can satisfy himself of the difference between the position of the absorption bands of Hb-O<sub>2</sub> and Hb-CO, he can always obtain a sample of Hb-O<sub>2</sub> for comparison with an unknown solution by pricking his finger.

296. Take a portion of the diluted solution of CO-haemoglobin just examined, treat it with a few drops of ammonium sulphide, warm in a bath at 50° C. for three minutes and examine with the spectroscope. No change takes place in the spectrum. (**Distinction from oxyhaemoglobin.**)

297. In two test-tubes place 2 or 3 cc. of solutions of oxyhaemoglobin and CO-haemoglobin of the same depth of colour. Fill the test-tubes with water and mix well. Note that the CO-

haemoglobin takes on a well-marked carmine tint, whilst the oxyhaemoglobin turns yellow. This simple test, which can only be seen on extreme dilution, rapidly serves to distinguish the two compounds.

298. **Methaemoglobin.** To 5 cc. of water add four drops of defibrinated blood. To the strong solution of oxyhaemoglobin thus formed add two drops of a saturated solution of potassium ferricyanide. The colour of the solution changes to a chocolate-brown. Examine with the spectroscope: there is visible a prominent band in the red, with its centre at about  $\lambda$  630. There is marked absorption of the blue end of the spectrum. Dilute with an equal bulk of water and examine again: two faint bands appear in the green in the position of the bands of oxyhaemoglobin.

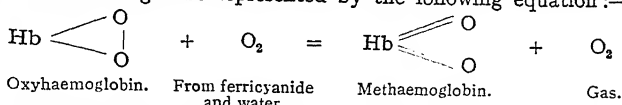
299. Dilute the solution of methaemoglobin thus obtained with another volume of water. Treat 5 cc. of this with two or three drops of ammonium sulphide and examine immediately. The colour changes to a red: the absorption band in the red disappears, and the spectrum of oxyhaemoglobin is seen. Warm the solution to 50° C. and allow it to stand for a short time (possibly with the addition of another drop or two of the reducing reagent). The two bands give place to the single band of reduced haemoglobin. Shake with air: oxyhaemoglobin is reformed.

300. Take a few cc. of defibrinated blood in a test-tube, add an equal quantity of water and warm to 50° C. to luke the blood. Oxygenate the solution by shaking with air, adding a drop of caprylic alcohol to prevent undue frothing. To the solution thus obtained add an equal bulk of saturated potassium ferricyanide. Mix by giving one shake, and then hold the tube at rest in an oblique position for a short time. Note the bubbles of gas (oxygen) that are evolved.

NOTES.—1. Oxyhaemoglobin is converted into methaemoglobin by the action of oxidising reagents, such as ferricyanides, nitrites, chlorates, and permanganates, and in the body, by the action of many aromatic substances, such as phenol.

2. The reaction is peculiar in that an amount of oxygen is evolved equivalent to that held in combination by the oxyhaemoglobin, although methaemoglobin contains the same percentage of oxygen as oxyhaemoglobin. This reaction is the basis of the modern method of estimating the amount of oxygen in the blood.

The reaction might be represented by the following equation :—



The oxygen is represented as being in a different state of combination in methaemoglobin, since it cannot be removed by submitting the compound to a vacuum.

3. When methaemoglobin is treated with a reducing reagent, the first change that occurs is that the linkage of the oxygen atoms reverts to that of oxyhaemoglobin; later, the oxygen is removed and reduced haemoglobin formed.

301. **Acid haematin.** To 5 cc. of water add four drops of defibrinated blood and five drops of strong acetic acid and heat. The colour changes to brown; and the solution shows an absorption band in the red, which is further from the D line than that of methaemoglobin. Its centre is about  $\lambda$  650.

302. **Acid haematin in ethereal solution.** Treat a few cc. of defibrinated blood with one drop of strong hydrochloric acid and a few cc. of acetic acid: extract this with about 5 cc. of ether by gentle shaking, pour the ether into a clean tube and examine it with the spectroscope. There is a prominent band in the red (centre  $\lambda$  638); on dilution with ether three other bands can be seen; a very narrow one with centre  $\lambda$  582; a broad one stretching from about  $\lambda$  555 to  $\lambda$  530 and another from  $\lambda$  512 to  $\lambda$  498.

303. **Alkaline haematin.** To 5 cc. of alcohol add 3 drops of 40 per cent. soda and 2 drops of defibrinated blood. Mix and heat till the alcohol just boils. Add an equal volume of water and examine spectroscopically. A prominent band is seen in the red, stretching from  $\lambda$  620 to  $\lambda$  595. On the blue-ward side is a shading extending to about  $\lambda$  570. The blue end of the spectrum may be absorbed.

304. **Alkaline haematin in alcohol.** Mix defibrinated blood into a thin paste with solid potassium carbonate and evaporate to complete dryness on a water bath. Powder the residue, boil with alcohol in a flask on the water bath and filter. The solution contains alkaline haematin free from proteins. It shows the absorption band of alkaline haematin more distinctly than the solution prepared in Ex. 303.

305. **Haemochromogen** (reduced alkaline haematin). Prepare a solution of alkaline haematin from dilute oxyhaemoglobin

as in Ex. 303. Treat 5 cc. with a few drops of ammonium sulphide. The colour of the solution changes to red. Examine with the spectroscope. Two absorption bands are seen in the green. The band nearer the D line (the  $\alpha$  band) is very prominent and sharply defined, with its centre at about  $\lambda$  558. The  $\beta$  band is much fainter and has its centre at  $\lambda$  520.

306. To 5 cc. of water add one drop of defibrinated blood and three or four drops of ammonium sulphide. Mix and warm cautiously till the oxyhaemoglobin has been completely reduced. Add a few drops of 40 per cent. soda and note the instantaneous formation of haemochromogen.

NOTE.—In very dilute solutions only the  $\alpha$  band can be seen. The absorption of light in this region is so intense that if a solution of oxyhaemoglobin, so dilute that its absorption bands cannot be readily seen, be converted by appropriate means into haemochromogen, the  $\alpha$  band of this pigment is usually observable.

307. **Acid haematoporphyrin.** To a few cc. of concentrated sulphuric acid in a test-tube add two drops of defibrinated blood (see note to Ex. 308) and mix by gentle shaking. Note the rich purple colour of the solution. Examine with the spectroscope. Two bands are seen: the  $\alpha$  band, with centre at  $\lambda$  600, being fainter and narrower than the  $\beta$  band, centre  $\lambda$  554.

308. **Alkaline haematoporphyrin.** To the solution of acid haematoporphyrin just prepared add five or six more drops of defibrinated blood, shaking gently after the addition of each drop. Pour the strong solution into about 50 cc. of cold water in a beaker, stir well and note the precipitate that rises to the surface. Transfer this precipitate to a test-tube by means of a rod; treat it with a few cc. of alcohol and boil. Add 5 cc. of sodium hydroxide. A solution of alkaline haematoporphyrin is thus obtained, which examined spectroscopically, after suitable dilutions, shows a four-banded spectrum. The centres of the bands are at  $\lambda$  622,  $\lambda$  576,  $\lambda$  539 and  $\lambda$  504 approximately.

NOTE.—The conversion of blood pigment into haematoporphyrin involves two processes. Firstly, the acid splits off the protein constituent (globin) and forms acid haematin; secondly, the acid haematin loses its iron and becomes haematoporphyrin. The first change is effected very readily even by dilute acids, but the separation of the iron from the haematin normally requires highly concentrated mineral acids. It has, however, been shown by Laidlaw that if the blood be first reduced the iron is split off with much greater ease by the acid. An efficient method of reducing defibrinated blood is that of "auto-reduction," in which a tightly corked vessel full of blood is allowed to stand for

a few days. If Exercises 307 and 308 be carried out with this reduced blood, care being taken by use of a pipette to prevent re-oxidation, the haemoglobin is entirely converted into haematoporphyrin, no trace of the brown haematin being left.

It sometimes happens that on pouring the acid solution into water a precipitate is not obtained. In such cases it is necessary to repeat the experiment, but to pour the acid haematoporphyrin into a smaller volume of water. On making a portion of this alkaline with 40 per cent. soda the spectrum of alkaline haematoporphyrin can generally be observed. It is sometimes necessary to examine such a solution in a thick layer, as in a beaker.

### 309. Preparation of haemin crystals.

A small drop of blood is spread to form a film on a glass slide and *slowly* evaporated till it is quite dry. To the film add two drops of a 0.1 per cent. solution of potassium chloride in glacial acetic acid. Cover with a slip and heat over a *very small* flame till bubbles appear and the solution is *boiling*. Immediately allow a drop or two more of the reagent to run under the cover slip and examine under a microscope.

NOTE.—Haemin is the di-acetyl ester of haematin hydrochloride. The above method is an extremely simple one of obtaining good specimens of the crystals, the production of which was formerly used as a test for blood in cloth, etc. It is important not to burn the blood during the drying process, and also to be sure that the acetic solution is rapidly brought to the boiling point.

The test can be applied to dilute solutions of haemoglobin by acidifying with acetic acid, precipitating with freshly prepared tannic acid, and treating the dried precipitate in a slide as described above. Suspected blood stains on linen, instruments, etc., should be extracted with a little alkali, the solution evaporated to dryness and treated as above.

## D. Blood constituents and their analysis.

**Glucose.** Human blood contains about 0.12 per cent of glucose. The available evidence indicates that this exists in a free state in the blood, and that it is equally distributed between the plasma and the corpuscles. The concentration in the blood increases after the ingestion of considerable amounts of glucose or cane sugar, but only increases after a meal of starch if the subject is abnormal. The extent to which the blood sugar rises after taking sugars depends on the rate at which the tissues, especially the liver, can assimilate the carbohydrate. If the blood sugar rises beyond 0.12 per cent. the condition is known as *hyperglycaemia*. This is generally followed by the excretion of easily detectable amounts of glucose in the urine, or *glycosuria*, the severity of which varies with the degree of

hyperglycaemia, and also on the permeability of the kidney to glucose. It must be noted that in certain individuals the kidney is abnormally permeable to sugar, so that marked glycosuria may exist without hyperglycaemia. This condition is known as "renal diabetes," or "diabetes innocens," this latter term being applied because it is not associated with the evil effects of the other types of diabetes. The rate at which the liver can assimilate sugar, or the "tolerance for sugar," is partially dependent on the activities of various organs, such as the pituitary, suprarenal and thyroid glands. Tolerance is best determined by blood analyses at appropriate intervals after administration of glucose: the amount in the urine being dependent on the renal permeability.

**310. Detection of glucose in blood.** Into a small flask measure 15 cc. of distilled water. Add 3 cc. of fresh defibrinated blood obtained from a slaughter house. Mix to luke the corpuscles. Heat to boiling, and keep the fluid boiling for a few seconds. Add 4 cc. of 1.25 per cent. "colloidal" ("dialysed") iron, adding it drop by drop and constantly agitating the flask. The proteins are completely precipitated. Add a "knife point" of solid potassium sulphate (to precipitate any excess of iron), and shake till it has dissolved. Filter through a small paper. To 5 cc. of the clear filtrate apply Cole's test for glucose (Ex. 104). A distinct positive test is obtained. To another portion of the filtrate apply the picric acid test (Ex. 108), controlling the latter by doing a blank test with an equal volume of water.

NOTES.—1. It is instructive to perform similar experiments on blood which has been incubated at 37° C. for 24 hours, toluol being added as an antiseptic. It will be found that the sugar has disappeared, the "glycolysis" being due to the action of special oxidising enzymes.

2. For the action of colloidal iron in precipitating proteins see page 10.

**311. The estimation of sugar in blood (Benedict's method).\***

*Principle.* The blood is laked, treated with picric acid and filtered. An aliquot part of the protein-free filtrate is heated with alkali. The glucose reduces some of the picric acid to picramic acid (Ex. 108), the amount of which is estimated in a colorimeter against a suitable standard.

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\* *Journ. of Biological Chemistry*, xxxiv., p. 203 (1918).

**Solutions and Apparatus required:**

1. *Picric-picrate mixture.* To 125 cc. of N. soda in a 1 litre measuring flask add about 700 cc. of hot distilled water, and then 36 grams. of pure, dry picric acid. Wash in with a little water, shake at intervals till dissolved, cool thoroughly, make up to 1 litre with distilled water, mix and filter if the solution is not crystal clear.

It is important to use pure *dry* picric acid. It is advisable to recrystallise the picric acid of commerce from boiling water, filtering the hot saturated solution through a hot water funnel. The cold solution is filtered through a Buchner funnel and the crystals spread out on layers of filter paper in a warm room till quite dry. The mother liquors can be used for the preparation of the stock solution of glucose mentioned below. It must be remembered that picric acid is an explosive, and that it is not safe to grind it in a mortar, or to send it through the post without damping it.

2. *Standard solution of glucose.* The stock solution required is a 1 per cent. solution in saturated picric acid. This can be prepared by dissolving 1 gram. of pure glucose (previously dried in a vacuum desiccator) in cold saturated picric acid and making the volume up to 100 cc. with the same solution. Or a strong (10 per cent.) solution can be estimated accurately by means of a polarimeter and a volume that contains exactly 1 gram. diluted to make 100 cc. with the saturated picric acid.

From this stock (which keeps for a considerable time) is prepared daily a solution which contains 0.64 mg. in 1 cc., by measuring 3.2 cc. into a 50 cc. flask and making up to the mark with distilled water.

An alternative standard is prepared from pure picramic acid, should this be available.\* The stock solution contains 100 mg. of picramic acid and 200 mg. of anhydrous sodium carbonate per litre. 126 cc. of this solution are treated with 1 cc. of 20 per cent. sodium carbonate solution and 15 cc. of the picric-picrate mixture and diluted to 300 cc. with distilled water. This solution exactly matches the colour from 0.64 mg. of glucose when treated in the way described below and diluted to make 12.5 cc. The picramic acid standard is not heated with the blood sugar.

3. *Sodium carbonate solution.* Dissolve 200 grams. of pure anhydrous sodium carbonate in hot distilled water and make the volume up to 1 litre. Filter when cold. The solution should be kept in a warm room, as it may crystallise out if the temperature gets very low.

4. Test-tubes graduated at 12.5 and 25 cc. A suitable internal diameter is  $\frac{3}{8}$  inch. It is convenient to have one tube engraved with "S" for the standard and one with "B" for the blood.

5. A 25 cc. volumetric flask. If this is not to hand, one of the graduated tubes can be used.

6. Ostwald pipettes of 1 and 2 cc. (See fig. 51.)

7. A colorimeter. (See p. 388.)

*Method.* 2 cc. of blood are drawn into an Ostwald pipette, containing a trace of powdered potassium oxalate to prevent coagulation. The blood must be drawn under strict antiseptic precautions. Discharge the blood into a 25 cc. volumetric flask (or graduated tube), and wash the pipette out twice with distilled water, adding the washings to the flask. After standing for a couple of

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\* For a method of preparation see Egerer, *Journ. of Biological Chemistry*, xxxv., p. 565.

minutes, with *gentle* agitation, to take the blood, add the picric-picrate mixture to the mark, and thoroughly shake the mixture. After standing for a minute or two\* the mixture is poured on to a dry filter and the clear filtrate collected in a small dry flask. Measure 8 cc. of the filtrate into one of the "B" calibrated tubes, add 1 cc. of the sodium carbonate solution, mix, and plug with cotton wool. If glucose is being used as a standard, measure 1 cc. of the diluted solution mentioned above (*i.e.* 0.64 mg. glucose) into one of the "S" tubes, using an Ostwald pipette. Add 3 cc. of distilled water, 4 cc. of the picric-picrate mixture and 1 cc. of the sodium carbonate. Mix and plug with cotton wool. Have a beaker or can of water boiling. Immerse both tubes in this and note the time. After exactly ten minutes' heating in the bath, the water of which must be kept boiling the whole time, remove the tubes and cool thoroughly. Dilute the standard to the 12.5 cc. mark with distilled water. Dilute the "B" tube to the same mark and compare the colours of the two solutions. If that of "B" is much greater than that of "S," the "B" tube can be treated with 2.5, 5, 7.5, 10, or 12.5 cc. of water, until the colours of the two solutions appear to be about the same, making a note of the amount of water added. The same procedure is adopted if the standard solution of picramic acid is used. The two solutions are now compared in a colorimeter (see p. 385), the standard being set at 15 mm.

*Calculation.* 2 cc. of blood are taken, diluted to 25 cc. and 8 cc. of the filtrate taken. The amount of blood actually used for the test is therefore  $\frac{8}{25} \times 2 = 0.64$  cc.

Since the colorimeter readings are inversely proportional to the concentrations of glucose

$$\frac{\text{mg. glucose in 0.64 cc. blood}}{0.64 \text{ (i.e. mg. glucose in standard)}} = \frac{\text{Reading of "S"}}{\text{Reading of "B"}}$$

$$\text{So glucose in 1 cc. of blood} = \frac{\text{Reading of "S"}}{\text{Reading of "B"}} \times \frac{0.64}{0.64} \text{ mg.}$$

$$\text{So gram. of glucose in 100 cc. blood} = \frac{\text{Reading of "S"}}{\text{Reading of "B"} \times 10}$$

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\* The method can be interrupted at this stage, the picric acid preventing glycolysis.



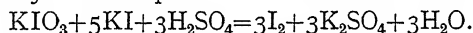
This is for a dilution of "B" to 12.5 cc. Should the dilution exceed this, a correction must be applied. Thus, if 7.5 cc. of water are added beyond the 12.5 mark, the result must be multiplied by

$$\frac{12.5 + 7.5}{12.5} = \frac{20}{12.5} = 1.6.$$

NOTE.—Benedict thinks that in advanced nephritis it might be necessary to modify the procedure owing to the presence of interfering substances, such as creatinine. His original paper should be consulted for details.

### 312. The micro-estimation of sugar in blood (Cole's method).

*Principle.* The proteins are precipitated by means of metaphosphoric acid. An aliquot part of the filtrate is boiled under standard conditions with a measured amount of an alkaline copper solution containing potassium iodate. A certain amount of the copper is thereby reduced to the cuprous condition. Sulphuric acid is added whilst the solution is still boiling. The cuprous compounds are almost instantaneously oxidised by the iodic acid, the evolution of CO<sub>2</sub> preventing any loss by the action of atmospheric oxygen. After cooling a small amount of potassium iodide is added. This reacts with any undecomposed iodic acid to form free iodine.



The iodine is titrated with N/<sub>200</sub> thiosulphate, soluble starch being used as an indicator. A blank determination of the amount of thiosulphate required for the given amount of the alkaline copper-iodate mixture having been made, the difference of the two readings is a measure of the amount of iodate lost and so of the sugar present in the blood. The sugar concentration is obtained by means of a curve, plotted from results obtained by the author with pure glucose.

#### Reagents, etc., required.

1. *Metaphosphoric acid.* Scrape a stick of glacial phosphoric acid and powder it in a clean, dry mortar. Have a weighed or tared test-tube ready, collect the powdered acid in this and weigh carefully. Measure out 5 times this weight of distilled water into a wide-mouthed flask, tip the acid into this and shake by a lateral movement until solution is complete.

This solution is stable for two or three days, and is a better precipitant after it has stood for an hour or so than when freshly prepared. When just made the precipitation of the proteins is not instantaneous, and in such cases it may be necessary to allow the mixture to stand for a short time before it is filtered.

#### 2. *Alkaline copper iodate mixture.*

|                             |    |    |    |           |
|-----------------------------|----|----|----|-----------|
| Potassium bicarbonate       | .. | .. | .. | 24 gms.   |
| Potassium carbonate         | .. | .. | .. | 36 gms.   |
| Crystalline copper sulphate | .. | .. | .. | 1.05 gms. |

Dissolve the bicarbonate in about 200 cc. of distilled water, without heating beyond 40° C. Add the carbonate, and when most of it has dissolved add the copper sulphate dissolved in about 20 cc. of water. Or add 15 cc. of a stock 7 per cent. solution. The copper must be added slowly with constant stirring. Now add the iodate, 9 cc. of a stock 1 per cent. solution. Make the volume up to 300 cc. with water, mix thoroughly and filter if necessary.

It is of the greatest importance to use the purest chemicals obtainable.

The solution should be kept in a well-stoppered bottle to avoid concentration. In this way it is apparently indefinitely stable.

3. *Thiosulphate solution.* Dissolve 54 gms. of pure crystalline sodium thiosulphate in recently boiled-out, cold distilled water and make the volume up to 2 litres. It is advisable to store this in a cool, dark cupboard for some days before standardisation. To standardise the stock solution, dissolve about 2 gms. of pure potassium iodide in about 5 cc. of water contained in an Erlenmeyer flask. Add about 5 cc. of a 1 per cent. solution of pure potassium iodate and then measure 20 cc. of accurately standardised sulphuric or hydrochloric acid, between 0.05 and 0.15 N. Titrate with the thiosulphate from a burette until the yellow colour has nearly disappeared. Then add a few drops of the solution of soluble starch (see below) and complete the titration. If  $x$  cc. of thiosulphate be required and the normality of the acid

is  $a \times N$ , the thiosulphate is  $\frac{20 \times a}{x} N$ . This should be between 0.106 and

0.108 N. Call it  $s \times N$ . Preparation of  $\frac{N}{200}$  thiosulphate from the stock.

Measure 100 cc. of recently boiled-out, cold, distilled water into a dry stoppered bottle by means of a 100 cc. pipette. Add 5 cc. of the stock thiosulphate, using a standard pipette. Add a further amount of water to make the

solution exactly  $\frac{N}{200}$ . The total volume of the solution should now be

$100 \times S$  cc. Thus, suppose the stock solution is 0.1074 N., the total volume is 107.4, consisting of 100 cc. water, 5 cc. of the thiosulphate and a further 2.4 cc. of water. This solution is well mixed and is perfectly stable, provided access of CO<sub>2</sub> and light be avoided. It is perhaps advisable to prepare it each day from the stock, but it can be safely stored in a cool, dark cupboard if the flask containing it be stoppered with a well-fitting rubber cork carrying a soda-lime tube.

4. *Potassium iodide*, 10 per cent. This is best kept in a bottle, stoppered with a rubber cork carrying a Dreyer's dropping pipette (Fig. 5).

5. *Soluble starch.* A 1 per cent. solution is prepared in the manner described for starch paste (p. 120). It is advisable to distribute the hot solution into several tubes and to plug these with cotton wool. If the tubes be immersed in a boiling water bath for ten minutes in each of three successive days, the starch will remain fit for use for many weeks. This avoids the necessity of having to prepare the solution whenever it is desired to make a blood sugar determination. For the preparation of soluble starch from potato starch, see p. 395.

6. Pure sulphuric acid, 10 per cent. by volume.

7. *A standard heating apparatus.* An apparatus similar to that shown on page 136 may be employed, a microburner being substituted for the Meker burner, and a piece of ordinary iron gauze for the asbestos gauze. But Bang's apparatus (slightly modified) is preferable. The pattern used by the author is shown in fig. 31. The bottle employed is one in which 2 lbs. of stick soda is usually supplied. A has an internal diameter of about 6 mm.; B about

4 mm. The lower end of A is about 30 mm. below the surface of the water in the bottle. This level is kept constant by attaching C to a suction pump, and B to a beaker of water. On turning on the pump and squeezing the tube at the far end of A the water finds the level of the lower end of C. This should be attended to daily if the apparatus is in constant use. From the far end of A the gas is conducted to a Y-piece and thence by rubber tubes to two "brewers burners," the orifices of which have been somewhat enlarged by unscrewing the tube and drilling with a fine knife point. These burners are placed under wire gauzes supported on moveable rings, the gauzes being about 30 mm. from the tops of the burners. When the gas is turned on, some of it bubbles through the water and escapes through B (where it is burnt), whilst the remainder passes to the burners. The amount passing to the burners is regulated by the water level, and is only slightly affected by the pressure of the gas past D. The apparatus is adjusted by trial till with a moderate size flame at B, 23 cc. of cold water in a 100 cc. Erlenmeyer flask are brought to the boil in two minutes. Once adjusted, the heating power is constant provided that the water level is constant and the flame at B is kept about the same. (The sketch shows this much too small).

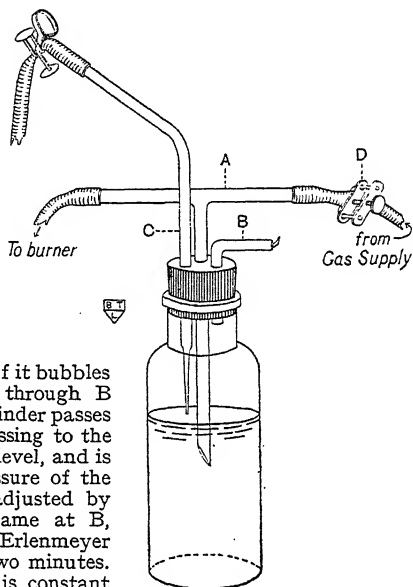


Fig. 31.

8. *Pipettes* graduated to contain 0.2 cc. The best form is made of narrow bore tubing without a bulb. It is more difficult to wash the last traces of the blood out of bulb pipettes than those with straight sides.

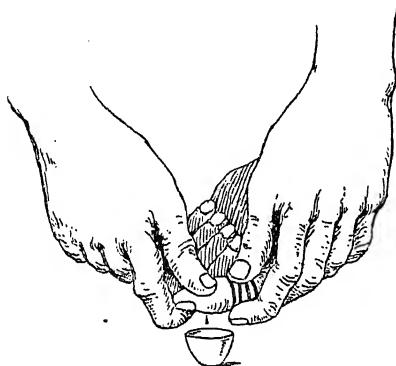


Fig. 32.

*Method of drawing the blood.*

A trace of finely powdered potassium oxalate is placed in the bottom of a small dry porcelain crucible. The left hand of the subject is washed in hot water and then dried. He is instructed to swing the arm vigorously backwards and forwards, keeping the hand as low as possible. A piece of  $\frac{1}{4}$  in. rubber tubing is then wound rather tightly round the finger five or six times, the first turn being made round the proximal joint and the others round the middle joint, so as to drive the blood towards the tip

of the finger. The back of the finger is then smartly jabbed with a

sterilised sharp, bayonet-pointed, probe or surgical needle about one-eighth inch above the nail. The operator then places his right thumb on the ball of the finger and his fore-finger on the nail, and by pressing firmly squeezes the blood out into the crucible, which may be held by the disengaged hand of the subject. The crucible should be gently rotated as the blood drops into it to ensure complete admixture with the trace of oxalate present. The rubber bandage should be removed as soon as sufficient blood has been collected. It is generally advisable to draw enough blood for two estimations, especially in the case of diabetic patients.

*Method of analysis.*

1. Measure 17.8 cc. of distilled water from a burette into each of two clean, dry, labelled test-tubes (6 by  $\frac{3}{4}$  in.).
2. Draw the blood as described above.
3. Suck up blood into one of the special 0.2 cc. pipettes mentioned above until it is just above the mark. Clean the end of the pipette carefully with a piece of filter paper and then allow the blood to run exactly to the mark. This is best done by holding the pipette almost horizontal.
4. Place the tip of the pipette about half-way down the water in one of the tubes and expel the blood by blowing very gently. Suck up water from the upper layer and discharge this back again, repeating this three or four times till the whole of the blood has been washed out. (Wash out the pipette repeatedly with cold or warm water before it has had time to dry. Then wash out with a mixture of equal parts of alcohol and ether, and dry thoroughly by means of a suction pump.)
5. Mix the contents of the tube by spinning it carefully between the palms of the hands.
6. Add exactly 2 cc. of the metaphosphoric acid solution, seal the tube with the thumb and shake well.
7. Measure another portion of 0.2 cc. of blood with another dry pipette (or the same one cleaned and dried as described above, but taking great care that every trace of ether and alcohol is removed before use) into the other labelled tube. Treat this as described in 5 and 6, reserving it for a duplicate analysis or for the main analysis should there be pronounced hyperglycæmia.
8. Filter off the coagulated proteins through a dry, sugar-free, 9 cm. No. 1 Whatman filter paper into a clean, dry test-tube.

9. Measure 15 cc. of this filtrate (which must be crystal clear) into a small (100 cc.) Erlenmeyer flask. Add exactly 5 cc. of distilled water and then 3 cc. of the alkaline copper iodate mixture. The latter must be measured with the greatest care, and the pipette allowed to drain for 15 seconds and then blown out whilst drawing it up the wall of the flask. It is essential that the same pipette be used for the blank titration (below) as for the actual estimation.

10. Mix the contents by careful agitation and place the flask on the centre of the heated gauze of the heating apparatus. If this has been correctly adjusted the solution should begin to boil in 2 minutes. Note the exact time when boiling commences. Allow the active boiling to continue for exactly 8 minutes, so that the heating should be ten minutes in all.

11. Just before the time is completed have 5 cc. of 10 per cent. sulphuric acid ready in a pipette. Allow a drop or two of this to run down the flask into the boiling mixture at the exact end of the 8 mins., and with the other hand remove the flask from the heater. Run the remainder of the acid down the side of the flask, gently agitating the latter.

12. Allow the mixture to stand for 2 minutes, and then *cool thoroughly* under the tap.

13. Add two drops of the 10 per cent. potassium iodide with the dropping pipette.

14. Titrate with  $N_{/200}$  thiosulphate from a 5 cc. micro-burette. When the yellow colour due to the iodine has nearly disappeared (normally after the addition of about 3.5 cc.) add two drops of the soluble starch and complete the titration, as indicated by the disappearance of the last trace of blue colour. This colour may slowly return, but no notice should be taken of this.

*Calculation.* The blank test is done by adding 2 cc. of the metaphosphoric acid to 18 cc. of distilled water, measuring 15 cc. of this into a 100 cc. Erlenmeyer flask and then proceeding as in 9 to 13 above. The figure for the blank should hold good for a considerable time, but it must be taken afresh with every batch of the copper iodate mixture or of stock thiosulphate. It is essential that the same pipette be used in the same way on every occasion.

The amount of thiosulphate required for the blood estimation

is deducted from that required for the blank. From this " $N/_{200}$  thiosulphate deficiency" the mgms. of glucose present in the 15 cc. of filtrate taken and the percentage of glucose in the blood can be read off from the curve shewn in fig. 33.

| cc. Thio-sulphate deficiency | Mgms. glucose present | Percentage in blood | cc. Thio-sulphate deficiency | Mgms. glucose present | Percentage in blood |
|------------------------------|-----------------------|---------------------|------------------------------|-----------------------|---------------------|
| 0.3                          | 0.0555                | 0.037               | 1.3                          | 0.213                 | 0.149               |
| 0.4                          | 0.074                 | 0.048               | 1.4                          | 0.240                 | 0.160               |
| 0.5                          | 0.09                  | 0.060               | 1.5                          | 0.256                 | 0.171               |
| 0.6                          | 0.105                 | 0.07                | 1.6                          | 0.273                 | 0.182               |
| 0.7                          | 0.121                 | 0.082               | 1.7                          | 0.29                  | 0.193               |
| 0.8                          | 0.138                 | 0.092               | 1.8                          | 0.306                 | 0.204               |
| 0.9                          | 0.156                 | 0.104               | 1.9                          | 0.322                 | 0.215               |
| 1.0                          | 0.172                 | 0.115               | 2.0                          | 0.343                 | 0.229               |
| 1.1                          | 0.189                 | 0.126               | 2.1                          | 0.357                 | 0.238               |
| 1.2                          | 0.207                 | 0.138               | 2.2                          | 0.372                 | 0.248               |

NOTES.—1. If the percentage of glucose is greater than 0.25, the other portion of blood mentioned above (in 7) is treated with 18 cc. of water and 2 cc. of the metaphosphoric acid. After mixing well and filtering the estimation is carried out as described, the result being multiplied by 2.

2. After the addition of the metaphosphoric acid the mixture can be stoppered and kept for the completion of the analysis for several days without affecting the result.

### 313. The estimation of chlorides by Foster's modification of the McLean—Van Slyke method.

*Principle.* The proteins are coagulated by means of metaphosphoric acid. The chlorides in the filtrate are precipitated by the addition of an excess of a standard solution of silver nitrate in nitric acid. To a given fraction of the filtrate from this precipitation, a solution of tri-sodium citrate is added equivalent in amount to the nitric acid present. This is to give the optimum reaction for the subsequent titration. Nitrate and soluble starch are also added at the same time. The mixture is now titrated with a standardised solution of potassium iodide. This combines with

\* F. C. McLean and Donald Van Slyke, *Journ. Biol. Chem.* xxi., p. 361 (1915). G. L. Foster, *Ibid.* xxxi., p. 483 (1917).

the silver to form silver iodide. The first trace of excess of iodide is converted to iodine by the nitrous acid, and the end point is indicated by the blue reaction with the starch.

### Apparatus and Reagents Required.

1. *Standard silver nitrate.* Dissolve 5.812 gm. of pure fused silver nitrate in about 600 cc. of distilled water. Add 250 cc. of pure concentrated nitric acid (Sp. Gr. 1.42). After mixing and standing for some time, make the volume up to 1000 cc. with distilled water. If pure metallic silver is used, 3.688 gms. are dissolved in the 250 cc. of nitric acid and the solution diluted with distilled water to make 1000 cc.

1 cc. of the silver nitrate  $\equiv$  2 mg. NaCl.

2. *Sodium citrate, nitrite, starch mixture.*

|   |         |          |
|---|---------|----------|
| Sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 + 5\frac{1}{2}\text{H}_2\text{O}$ ) | ..      | 446 gm.  |
| Sodium nitrite  | .. .. . | 20 gm.   |
| Soluble starch  | .. .. . | 2.5 gm.  |
| Distilled water to  | .. .. . | 1000 cc. |

In preparing this it is essential that all the substances used be free from chlorides. If chlorides are present, the end point is very difficult to determine, and the process becomes unworkable.

Dissolve the starch (see p. 395) with the aid of heat in about 500 cc. of water. Add the citrate and nitrite and heat on the water bath till all is dissolved. It is not usually necessary to filter, the suspended impurities having no influence on the titration. When cool, make up to 1000 cc. with water.

3. *A standard solution of potassium iodide.* Dissolve 6.1 gm. of potassium iodide and make the volume up to about 2100 cc. with distilled water.

This is standardised as follows:—5 cc. of the silver nitrate are treated with 5 cc. of the citrate mixture and titrated with the iodide from a burette. A yellowish precipitate of silver iodide is formed, but the first drop in excess gives a distinct bluish tinge, which is taken as the end point. The next drop of iodide gives a marked deepening of the colour, but the first change is readily detected with a little practice. The iodide solution is then diluted so that 10 cc. are required for 5 cc. of the silver. Thus, if 9.24 cc. are used for the

preliminary titration, add  $\frac{(10-9.24) \times 1000}{9.24}$  cc. of distilled water to every litre of the solution.

2 cc. of this standardised solution  $\equiv$  1 cc. of the silver nitrate.

4. *Metaphosphoric acid.* See Ex. 312, p. 253.

5. *Magnesium sulphate*, 10 per cent.

*Drawing the blood.* The skin at the bend of the elbow is sterilised, a rubber tube is clamped rather tightly round the middle of the upper arm, and the patient is made to grip firmly a bandage or pad of cotton wool. A sharp sterile hollow needle is then passed into the prominent median basilic vein. The needle is attached to a short length of rubber tubing, the open end of which passes into a test-tube into which the blood is collected. Just before use the interior of the tube is slightly moistened by breathing into it. A small pinch of finely powdered potassium oxalate is added to the tube, and is distributed round the inner walls of the tube, adhering to them by reason of the trace of moisture. As the blood flows into the tube this is rotated by an assistant, so as to ensure a good admixture of the oxalate with the blood, thus preventing coagulation. When sufficient blood has been drawn, the tourniquet is removed, the patient is instructed to release his hold on the pad and the

needle is removed. The puncture is covered with a little collodion and cotton wool and rarely gives any trouble. In inserting the needle it is advisable to hold the vein firmly by the sterilised thumb and forefinger of the left hand, so as to prevent the vein from slipping away from the needle.

*Method of analysis.*

1. Add about 20 cc. of distilled water to a 25 cc. volumetric flask.

2. Add 2 cc. of the blood by means of a pipette graduated "to contain" 2 cc. After discharging the blood into the water, suck up water from the upper portion into the pipette and blow this back into the flask, repeating this process until the whole of the blood has been removed. Mix thoroughly by gentle rotation.

3. Add 1 cc. of the metaphosphoric slowly and with constant shaking. Fill to the mark with water and shake well.

4. Allow to stand for 10 minutes with occasional shaking, so that the chlorides are equally distributed throughout the mixture.

5. Filter through a dry 9 cm. No. 1 Whatman paper into a dry test-tube.

6. Measure 10 cc. of the clear filtrate into another 25 cc. volumetric flask.

7. Add 5 cc. of the standard silver nitrate, 5 cc. of the 10 per cent. magnesium sulphate (to flock the precipitate), fill to the mark with water, shake thoroughly, and allow to stand for 5 minutes.

8. Filter through a dry 9 cm. No. 1 Whatman filter paper into a dry test-tube. Should the first portions come through cloudy they must be passed through the paper again until a perfectly clear filtrate is obtained.

9. Measure 20 cc. of the filtrate into a small Erlenmeyer flask. Add 4 cc. of the sodium citrate mixture.

10. Titrate with the standard solution of potassium iodide from a 5 cc. microburette. The end point is marked by the first appearance of a blue green tint. Should this be overshot, add 1 cc. of the silver nitrate by means of an Ostwald pipette, then 1 cc. of the citrate mixture and complete the titration.

*Calculation.* Let  $x$  cc. be the amount of iodide required. 2 cc. of blood are made up to 25 cc. 10 cc. of this are taken in (6), corresponding to  $\frac{2 \times 10}{25} = \frac{4}{5}$  cc. blood. This is treated with 5 cc. of the silver and diluted to



25 cc. 20 cc. of the filtrate require  $x$  cc. of iodide, so 25 cc. would require  $\frac{25}{20}x$ . This corresponds to  $\frac{25}{20}x$  cc. of silver.

So amount of silver precipitated by the chlorides in 4 cc. of blood is

$$\left(5 - \frac{25x}{40}\right) \text{ cc.}$$

So  $\frac{4}{5}$  cc. blood contain  $\left(5 - \frac{25x}{40}\right) \times 2$  mg. NaCl.

And 100 cc. blood contain  $\left(5 - \frac{25x}{40}\right) \times 2 \times \frac{5}{4} \times 100$  mg. NaCl.

This simplified is  $156.25(8-x)$  mg. NaCl per 100 cc. or approximately  $156(8-x)$  mg. per 100 cc.

Should the end point be overshot and 1 cc. of extra silver added, the  $x$  in the above calculation is obtained by deducting 2 from the total amount of iodide used.

NOTE.—The filtrate obtained in the estimation of non-protein nitrogen (Ex. 314) can be used for the determination of chlorides. In this case 10 cc. of the protein free filtrate (containing the chlorides of 1 cc. of blood) is used for (6) as above. In this case the calculation is

$$125(8-x) = \text{mg. of NaCl per 100 cc. blood.}$$

### 314. The estimation of the non-protein nitrogen of blood.

**Principle.** Blood is treated with water and then with a solution of metaphosphoric acid, which precipitates the whole of the proteins (Ex. 17). An aliquot portion of the filtrate is concentrated and the total nitrogen determined by Kjeldahl's method.

#### Reagents and Apparatus Required.

1. The usual requisites for Kjeldahl's method (see p. 322).
2. Freshly prepared solution of metaphosphoric acid. Weigh out 2.5 grams. of "glacial phosphoric acid," and crush it in a mortar with 8 cc. of distilled water.
3. The apparatus shewn in fig. 34. A is a wash bottle containing 1 in 5 sulphuric acid, to remove ammonia from the air.
- B. A 300 cc. Kjeldahl flask.
- C. A funnel, with tap and side piece, as shewn.
- E. A plain condenser.
- G. A boiling-tube ( $10 \times 1\frac{1}{4}$  in.), with the flange ground off on a wet stone. The tubes B and G and the inner tube of E must be of resistance glass.
4. An ordinary Bunsen fitted with a rose-top.
5. A foot bellows or a blast pump to enable the final titration to be conducted in a  $\text{CO}_2$ -free atmosphere. The air is led through wash bottles containing 1 in 4 sulphuric acid (to remove ammonia) and then through soda (to remove the  $\text{CO}_2$ ). It then passes to a thick-walled tube, which is bent to fit the tube G, as shewn in fig. 35.

**Method.** To about 20 cc. of distilled water in a 50 cc. volumetric flask add 5 cc. of recently drawn blood and mix by gentle agitation. Add 3 cc. of the freshly prepared solution of metaphosphoric acid and mix. Full up to the mark with distilled water, mix and transfer

the contents to a 100 cc. flask. Shake vigorously at intervals for at least 5 minutes, or allow the mixture to stand for a longer period. Filter into a dry tube through a dry paper. The filtrate should be crystal clear; if it is not, it must be passed through the filter till clear. Transfer 20 cc. of the filtrate to the 300 cc. Kjeldahl flask (B), add 2 cc. of pure sulphuric acid, 1 gram of pure potassium sulphate

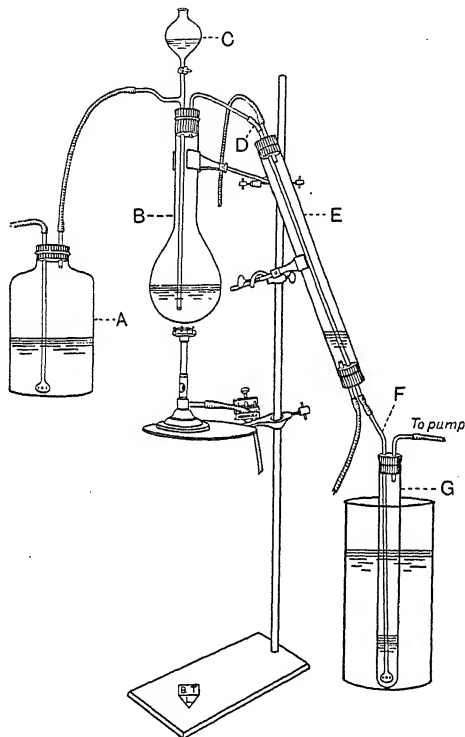


Fig. 34. Apparatus for Micro-Kjeldahl by Cole's modification.

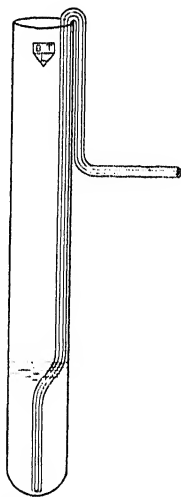


Fig. 35. Tube for titrating in a  $\text{CO}_2$  free atmosphere.

and 2 drops of saturated copper sulphate. Heat over a micro-burner, using a Folin fume-absorber (p. 383). A fairly large flame can be used at first, until the greater part of the water has boiled off. Care must be taken to see that the bulb of the fume-absorber is emptied if necessary. When the volume of fluid has been considerably reduced the flame should be adjusted so that the mixture boils

gently. The height of the flame may have to be slightly increased towards the end of the incineration. It is better to use a small flame nearly touching the flask than a larger one a few inches away. A screen to keep off draughts is sometimes necessary. After a time the fluid goes nearly black, and subsequently lightens in colour until it is a faint blue or green. Heating must be continued for at least 5 minutes after this stage has been reached.

Remove the flame and allow to cool until the flask can be held in the hand. Then add 20 cc. of distilled water and shake. If a solid cake separates out, the flask must be cautiously heated until this has completely dissolved. Add 10 cc. of rectified alcohol and connect up to the apparatus. Into tube G measure 10 cc. of standard sulphuric acid (about 0.04 N.), add 3 or 4 drops of methyl red and connect the tube up to the apparatus, the tube being as will be seen by the fluid becoming blue. Heat the solution with a Bunsen with a rose-top, to boiling point. The use of a rose-top minimises the risk of the tube cracking. The flame should not play directly on the glass. The air current and the boiling can be regulated to minimise the risk of splashing. The distillation should be allowed to continue for 10 to 11 minutes. Remove the flame, but do not stop the air current. Disconnect the rubber joint at D and wash the inner tube of the condenser down into G with a jet of water. Disconnect the pump and then the rubber connexion of F to the condenser. Remove the stopper of G with the tubes and wash down the interior and exterior of F into G. Fit the tube shewn in fig. 35 to G and titrate with standard CO<sub>2</sub>-free soda (which should be about 0.04 N.).

*Calculation and Example (see p. 323).*

Soda was 0.032 N.

Acid was 0.029 N. (1 cc. =  $0.029 \times 14 = 0.406$  mg. N.).

Soda required for titration was  $7.61 \text{ cc.} = 76.1 \times \frac{32}{29} = 8.40 \text{ cc. of the acid,}$

So acid neutralised by the Nitrogen in 20 cc. filtrate was  $10 - 8.4 = 1.6 \text{ cc.}$

So non-protein N. in 2 cc. blood =  $1.6 \times 0.406 = 0.65 \text{ mg.}$

Blank determination = 0.02 mg. N.

So non-protein N. in 2 cc. blood = 0.63 mg.

Non-protein N. in 100 cc. = 31.5 mg.

NOTES.—1. Folin and Denis (*Journ. Biol. Chem.*, xxvi., p. 491) introduced the method of precipitating the proteins with metaphosphoric acid. They estimate the nitrogen by direct Nesslerisation after incinerating with a mixture of sulphuric and phosphoric acids. The author has not been successful in repeating their results, being troubled with the rapid formation of a cloud after

adding Nessler's reagent. Under these circumstances the above method of distillation of the ammonia has been elaborated.

2. The amount of non-protein nitrogen in normal human blood seems to vary between 30 and 40 mgms. per 100 cc. It is increased in certain types of nephritis and in severe hepatic disease.

### 314A. The estimation of urea in blood.

*Apparatus, etc.* This is exactly the same as that shown in fig. 44, and the principle is the same as that for the estimation of urea in urine (page 335). The only additional requisites are a cold saturated solution of potassium carbonate and a pipette graduated to contain 3 cc.

*Method.* Into B measure 2 cc. of the 0.6 per cent. acid potassium phosphate and 2 cc. of distilled water. Add 3 cc. of blood (whole blood, plasma or serum) and wash this out of the pipette as completely as possible by means of the fluid in the tube. Add 5 drops of caprylic alcohol and 0.3 gm. of finely ground Soya bean meal. Into E measure 20 cc. of standard sulphuric acid (0.04 to 0.07 N) and add 2 drops of caprylic alcohol. Connect up the whole apparatus to the water pump, immerse B in a can of water at 45° C. and send a slow air current through the apparatus for 15 mins. Remove B from the water bath and send a strong air current through for 1 minute. Stop the pump, disconnect the entry and exit tubes of B and remove the stopper with these tubes and place it on the bench in such a way that none of the fluid is lost. Add 4 cc. of saturated potassium carbonate and then about 3 gm. of solid anhydrous potassium carbonate. Lightly shake, replace the stopper and tubes, connect up and send a rather slow air current through for two minutes and then the strongest possible current for 12 minutes. Stop the pump and proceed as directed on page 332 (iv.).

*Calculation.* The amount of preformed ammonia in the blood and that derived from the Soya meal can usually be neglected without sensible error.

Let  $a$  be the amount of acid employed and its normality  $A$ .

Let  $s$  be the amount of soda required for back titration and its normality  $S$ .

Then  $\left(a - \frac{s \times S}{A}\right) A \times 1000 = \text{mgms. urea per 100 cc. blood.}$

The average amount for normal blood is about 30 mgms. In severe cases of interstitial nephritis it often exceeds 100 mgms.

## CHAPTER XI.

### THE CONSTITUENTS OF BILE.

Bile is secreted continuously into the hepatic ducts by the liver. During the intervals of digestion it is stored in the gall bladder, being poured into the duodenum when the acid chyme passes through the pylorus.

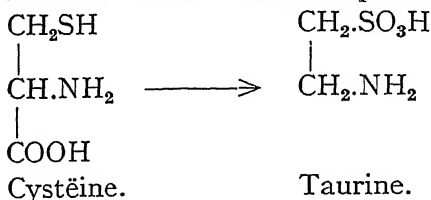
During its stay in the gall bladder there is an absorption of water and an increase in the protein content, resulting in an increase in the specific gravity from about 1010 to 1040.

The percentage composition of human bile varies considerably. The following are average figures:—

|                    | From<br>Gall Bladder. | From<br>Fistula. |
|--------------------|-----------------------|------------------|
| Water .. .. .      | 86                    | 98               |
| Solids .. .. .     | 14                    | 2                |
| Bile salts ..      | 9                     | 0.8              |
| Protein ..         | 3                     | 0.3              |
| Bile pigments }    |                       |                  |
| Cholesterol ..     | 0.2                   | 0.06             |
| Lecithin and fat   | 1.0                   | 0.04             |
| Inorganic salts .. | 0.8                   | 0.8              |

The bile salts are the sodium salts of glycocholic and taurocholic acids. They are formed by the condensation of cholalic acid ( $C_{24}H_{40}O_5$ ) with glycine (amino-acetic acid,  $CH_2.NH_2.COOH$ ) and taurine respectively. Glycine is one of the products obtained by the hydrolysis of proteins.

Taurine is derived from a similar product, cysteine.



The bile acids are hydrolysed into their constituents by boiling acids and also by the intestinal bacteria.

The bile salts are soluble in water and alcohol, insoluble in ether.

Their solutions have a remarkably low surface tension. (See Hay's test.)

They have the following functions:—

1. They have a marked adjuvant action on pancreatic lipase. (See Ex. 167.)
2. They are solvents for the fatty acids and markedly increase the absorption of fats.
3. They thus help to remove the fatty film surrounding the protein, and allow the proteolytic ferments to act. In this way, by assisting the absorption of proteins, they diminish bacterial decomposition. They are not direct antiseptics.

**Preparation of Bile Salts.**—Mix 40 cc. of ox gall with enough animal charcoal (about 10 grams.) to form a paste. Evaporate to dryness over a water bath, stirring at intervals. Grind the residue in a mortar, transfer it to a flask, add about 70 cc. of 96 per cent. or absolute alcohol and boil on the water bath for 20 minutes. Cool and filter into a dry beaker. Add ether to the filtrate till there is a slight permanent cloudiness. Cover the beaker with a glass plate and allow it to stand in a cool place for 24 hours. A crystalline mass of bile salts separates out. The crystals are filtered off and allowed to dry in the air.

For the following tests use diluted ox or sheep gall:—

315. **Pettenkofer's test for bile salts.** To 5 cc. of the solution add a *small* particle of cane-sugar and shake or warm till this has completely dissolved. To the cooled solution add 5 cc. of concentrated sulphuric acid, inclining the test-tube so that the acid settles to the bottom. Gently shake the test-tube from side to side. As the fluids gradually mix a deep purple colour develops.

NOTES.—1. This reaction depends on the production of furfural from the cane-sugar by the strong acid. (See Ex. 114.)

2. If too much cane-sugar be taken the fluid will turn brown or black, owing to the charring produced.

3. Proteins give a very similar reaction with furfural in the presence of strong acids. (See Ex. 26.) Proteins also tend to give a brown char with sulphuric acid. For these reasons it is advisable to remove the proteins from solution before attempting the test.

4. The purple colour obtained is only stable in the presence of strong sulphuric acid. It disappears on dilution with water.

5. If a small portion of the coloured fluid be diluted with 50 per cent. sulphuric acid, and examined with the spectroscope, two absorption bands will be seen, one between the lines C and D, nearer the latter; the other in the green, overlapping E and B.

6. The test cannot be applied directly to urine, owing to the presence of chromogenic substances that yield intense colours with sulphuric acid.

316. **Hay's test for bile salts.** Take 10 cc. of the solution in a test-tube. Sprinkle the surface with flowers of sulphur and note that they fall through the liquid to the bottom of the tube. Repeat the test with water, noting that the particles remain on the surface.

NOTES.—1. This test for bile salts depends on the remarkable property that they possess of lowering the surface tension of water, thus enabling the particles of sulphur to sink through the fluid.

2. The test is of great value for the detection of bile salts in urine.

3. This property of bile salts is utilised by draughtsmen in preparing tracings on oiled paper, on which ink collects in drops, and does not spread well. If the paper be first treated with a little ox gall and allowed to dry the difficulty is removed, owing to the reduction in surface tension.

4. A method for estimating bile salts in urine has been described by Grünbaum, depending on this property. The rate of escape of the urine from standard capillary tubes is noted, the rate increasing with the concentration of bile salts.

317. **Oliver's test for bile salts.** Acidify 5 cc. of the solution with two or three drops of strong acetic acid, filtering if necessary. To the acid solution add an equal quantity of 1 per cent. solution of peptone. A white milkiness or a decided precipitate is produced, insoluble in excess of acid.

NOTES.—1. The precipitate formed consists of a compound of protein with bile acids.

2. The test can be applied to urine. (Ex. 379.)

### The Bile Pigments.

Bilirubin,  $C_{32}H_{36}N_4O_6$ , is a reddish-brown pigment most abundant in the bile of carnivora. It is readily oxidised by the oxygen of the air into biliverdin,  $C_{32}H_{36}N_4O_8$ , the green pigment found mostly in the bile of herbivora. These compounds are formed in the liver cells from the

products of disintegration of haemoglobin. Haematin is  $C_{32}H_{32}N_4O_4Fe$ , and haematoporphyrin is isomeric with bilirubin.

They are weak acids, forming sodium and calcium salts, the latter being insoluble in water. Free bilirubin is soluble in ether and chloroform: the sodium compound is insoluble, as is free or combined biliverdin.

By oxidation bilirubin is converted, through a number of ill-defined bodies, such as bilicyanin, and bilifuscin, into choletelin, the end product of Gmelin's reaction.

By further oxidation a compound, haematinic acid ( $C_8H_8O_5$ ), is formed, identical with the product obtained by the oxidation of haematin or haematoporphyrin.

By reduction with sodium amalgam in alcoholic solution the bile pigments are converted into hydrobilirubin, which is also formed by the action of more powerful reducing agents on haematin or haematoporphyrin.

These facts all indicate the close relationship between haematin and the bile pigments.

In the bowel the bacteria first reduce bilirubin to hydrobilirubin. This is then attacked, two nitrogen atoms being probably removed, the result being the formation of urobilin, which is mainly excreted in the faeces, being sometimes called "stercobilin." A certain amount however, is absorbed into the blood, and excreted by the liver into the bile, whilst a small amount is excreted by the kidney in the form of urobilinogen. (See p. 278.)

### 318. Huppert-Cole test for bile pigments.

Boil about 15 cc. of the fluid in a test-tube. Add two drops of a saturated solution of magnesium sulphate, then add a 10 per cent. solution of barium chloride, drop by drop, boiling between each addition. Continue to add the barium chloride until no further precipitate is obtained. Allow the tube to stand for a minute. Pour off the supernatant fluid as cleanly as possible or use a centrifuge. To the precipitate add 3 to 5 cc. of 97 per cent. alcohol, two drops of strong sulphuric acid, and two drops of a 5 per cent. aqueous solution of potassium chlorate. Boil for half a minute and allow the



barium sulphate to settle. The presence of bile pigments is indicated by the alcoholic solution being coloured a greenish blue.

NOTES.—1. To render the test more delicate, pour off the alcoholic solution from the barium sulphate into a dry tube. Add about one-third its volume of chloroform and mix. To the solution add about an equal volume of water, place the thumb on the tube, invert once or twice and allow the chloroform to separate. It contains the bluish pigment in solution.

2. The bile pigment is adsorbed on to the barium sulphate precipitate, but passes into solution again in acid alcohol. The chlorate acts as a very weak oxidising reagent, converting bilirubin and biliverdin to the characteristic blue compound.

3. The author claims that it is a very much more delicate test than the one that follows.

319. **Gmelin's test for bile pigments.** Take a few cc. of fuming yellow nitric acid in a test-tube, and by means of a pipette carefully place on the surface of this an equal amount of bile. Shake the tube very gently from side to side, and note the play of colours in the bile as it becomes oxidised by the acid. Proceeding from acid to bile the colours are yellow, red, violet, blue, and green.

NOTES.—This test can be modified in many ways.

1. Add a drop of yellow nitric acid to a thin film of bile on a white porcelain plate. The drop of acid becomes surrounded by rings of the various colours.

2. Filter some diluted bile repeatedly through an ordinary filter paper, and then place a drop of fuming nitric acid on the paper.

**Cholesterol** has been described on p. 161, and **Lecithin** on p. 163.

### **The Protein of Bile.**

When bile is treated with acetic acid a precipitate is formed insoluble in excess. This was formerly thought to be mucin. But it has been shown that it is nucleoprotein, the bile salts present preventing the re-solution in strong acetic acid. (See Ex. 317.) In human bile, however, mucin is present as well as nucleoprotein.

The protein is secreted by the cells lining the ducts and the gall bladder, so that bile from the gall bladder contains a much greater percentage than fistula bile.

320. To a small quantity of undiluted bile add strong acetic acid, drop by drop. A precipitate is formed, insoluble in excess of acid. This precipitate consists of a nucleoprotein, together with a considerable amount of the bile salts and bile pigments

## CHAPTER XII.

### URINE AND ITS CHIEF CONSTITUENTS.

#### A. The average composition.

The composition of the urine varies with the individual and with the diet. Below we give the figures in grams. for the daily output of

- A. The average man on the average mixed diet.
  - B. An individual on a liberal diet.
  - C. The same individual on a diet deficient in proteins.
- B. and C. are taken from a paper by Folin.

|                           | A.   |           |                             | B.   |           |                             | C.   |           |                             |
|---------------------------|------|-----------|-----------------------------|------|-----------|-----------------------------|------|-----------|-----------------------------|
|                           |      | Nitrogen. | Per cent. of Total N. or S. |      | Nitrogen. | Per cent. of Total N. or S. |      | Nitrogen. | Per cent. of Total N. or S. |
| Urea                      | 30   | 14        | 87.5                        | 31.6 | 14.7      | 87.5                        | 4.72 | 2.2       | 61.7                        |
| Ammonia                   | 0.6  | 0.5       | 3.1                         | .6   | 0.49      | 3.0                         | .51  | 0.42      | 11.3                        |
| Creatinine                | 1.55 | 0.57      | 3.6                         | 1.55 | 0.58      | 3.6                         | 1.61 | 0.60      | 17.2                        |
| Uric Acid                 | 0.7  | 0.23      | 1.4                         | .54  | 0.18      | 1.1                         | .27  | 0.09      | 2.5                         |
| Undetermined              |      | 0.7       | 4.4                         |      | 0.85      | 4.8                         |      | 0.27      | 7.3                         |
| Total—N                   |      | 16.0      | 100.0                       |      | 16.8      | 100.0                       |      | 3.6       | 100.0                       |
| Inorganic SO <sub>3</sub> | 2.92 |           | 88.2                        | 3.27 |           | 90.0                        | 0.46 |           | 60.5                        |
| Ethereal SO <sub>3</sub>  | .22  |           | 6.6                         | 0.19 |           | 5.2                         | 0.10 |           | 13.2                        |
| Neutral SO <sub>3</sub>   | .17  |           | 5.2                         | 0.18 |           | 4.8                         | 0.20 |           | 26.3                        |
| Total SO <sub>3</sub>     | 3.3  |           | 100.0                       | 3.64 |           | 100.0                       | 0.76 |           | 100.0                       |

## B. The Physical Chemistry of the Urine.

### I. General Properties.

Normal human urine is a clear yellowish fluid, the depth of the tint depending largely on the concentration. On standing, a cloud (nubecula) of mucoid containing epithelial cells separates out. After a heavy meal urine may be passed cloudy, due to earthy phosphates and carbonates. On standing, these settle to the bottom of the vessel as a white deposit, insoluble on warming, but soluble in acids.

Also on standing a cloud of urates may settle as a reddish deposit that clears up on warming.

Fresh urine has a characteristic odour of the aromatic type, due to the presence of some substance that has not yet been recognised. On standing, an unpleasant ammoniacal odour develops as the result of bacterial decomposition.

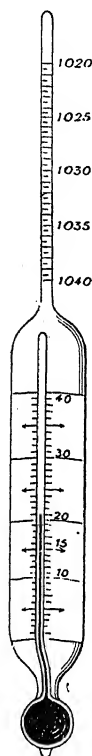
### II. The Specific Gravity.

Usually lies between 1012 and 1024 (water = 1000). With copious drinking it may fall to 1002. After excessive perspiration it may rise to 1040.

The determination of the specific gravity for clinical purposes is most conveniently made by means of a urinometer, a weighted cylinder that floats in the urine. The depth to which it sinks depends on the density of the fluid, and this can be read directly by means of a graduated scale on the stem. The instrument is calibrated for a certain temperature, usually 15° C.

The urine should be either cooled or warmed to this temperature, or a correction made by adding 1 unit for every 3 degrees above this, or subtracting 1 for every 3 degrees below the standard. Thus, if the reading be 1018 at 18° C., the corrected Sp. Gr. is 1019.

To obtain the best results two separate instruments should be at hand, the one calibrated from 1000 to 1020 and the other from 1020 to 1040.



The total amount of solids in the urine can be roughly calculated from the specific gravity by Long's coefficient. The last two figures of the specific gravity  $\times 2.6$  gives total solids in 1000 cc.

Thus specific gravity at  $25^{\circ}\text{C} = 1.017$ .

Total solids in 1000 cc.  $= 17 \times 2.6 = 44.2$  grams.

Häser's coefficient (2.33) on a similar basis, but calculated for  $15^{\circ}\text{C}$ . is probably inaccurate.

321. Take the specific gravity of normal urine by means of a urinometer. Wipe the instrument clean, and float it in the centre of a cylinder containing the urine. Remove all froth, by means of filter paper or by placing a single drop of ether on the surface of the urine. Take care that the instrument does not touch the sides of the vessel. Place the eye level with the surface of the fluid and read the division of the scale to which the latter reaches. Read the level of the true surface of the urine, not the top of the meniscus around the stem.

### III. The Osmotic Pressure (Cryoscopy).

The method of taking the freezing point of a fluid is described on p. 8, and the subject has been considered from a theoretical standpoint on p. 5.

Fig. 36.  
Urinometer.

In urine the concentrations of certain substances, such as urea, are much greater than they are in the blood. The work done by the kidney in effecting this concentration can be calculated from a consideration of the osmotic concentration, *i.e.*  $\Delta$ , of each substance in blood and urine. It is quite erroneous to imagine that the work done can be calculated from a knowledge of the total osmotic concentration of the blood and urine respectively. But, at the same time, the determination of  $\Delta$  of the blood and of the urine secreted by each kidney in certain renal diseases may

give us valuable information as to the relative activities of the two organs.

$\Delta$  of blood is about  $0.55^{\circ}$  C., the same as that of a 0.9 per cent. solution of sodium chloride.

$\Delta$  of urine varies considerably with the diet, volume of fluid taken and other conditions. For the mixed 24 hours urine of an average man it is usually about  $1.2^{\circ}$  C. The following values are of interest in this connection:—

$\Delta \times \text{volume of urine} = \text{molecular diuresis.}$

$\frac{\Delta}{\text{NaCl per cent.}}$  is of considerable pathological significance. It is fairly constant in health, varying between 1.25 and 1.6. It exceeds 1.7 in heart disease or in any condition that causes a retardation of the renal circulation. The only febrile condition in which it is less than 1.7 is malaria.

#### IV. Reaction.

Normal human urine is generally acid to litmus, the average  $P_H$  of 24 hour specimens being about 6.0. The reaction varies with the diet, being greatest on a meat diet, owing to the oxidation (in the body) of the sulphur and phosphorus to sulphuric and phosphoric acids. On a vegetable diet, however, the urine may become alkaline, as it is in herbivora, owing to the organic salts being oxidised to alkaline carbonates. During the secretion of the acid gastric juice into the stomach, the urine may become alkaline, the so-called "alkaline tide."

The acid reaction of the urine is mainly due to the excretion of acid phosphates and of weak organic acids. A certain amount of the acids produced in the body are neutralised by ammonia and excreted as ammonium salts in the urine. The ingestion of acids or of acid phosphates usually leads to an increase in the excretion of titratable acids (or acid salts) and of non-titratable neutral am-

monium salts. The sum of the two can be taken as a measure of the total amount of acid eliminated from the body. The titratable acid excreted is usually measured by titrating to phenol phthalein, but it is better to titrate to blood reaction, *i.e.*  $P_H = 7.45$ . By the use of the comparator described below, this is a very simple matter.

Palmer and Henderson\* have studied the relationships between reaction, volume of urine, etc. The following table gives some of the results obtained in apparently healthy subjects:—

| $P_H$                     | 24 hours<br>Volume in cc. | Titratable<br>Acid in cc. of<br>0.1 N Acid. | $NH_3$ in cc. of<br>0.1 N Acid. | A + N | $\frac{A}{N}$ |
|---------------------------|---------------------------|---|---------------------------------|-------|---------------|
|                           |                           | A   | N                               |       | R             |
| 5.4                       | 1026                      | 320   | 367                             | 687   | 0.87          |
| 5.7                       | 1193                      | 303   | 384                             | 687   | 0.79          |
| 6.0                       | 1259                      | 263   | 365                             | 628   | 0.72          |
| 6.6                       | 1400                      | 224   | 357                             | 581   | 0.63          |
| Average of all the cases. |                           |   |                                 |       |               |
| 5.94                      | 1231                      | 278   | 370                             | 649   | 0.75          |

They note that

1. A increases with the hydrogen-ion concentration.
2. With constancy in the excretion of phosphoric acid the hydrogen-ion concentration varies with A.
3. With constancy of A, the hydrogen-ion concentration varies as the phosphoric acid.
4. N is fairly constant. *Normally* the final regulation of the reaction through excretion falls upon the phosphates.
5. The volume increases as the acidity decreases.

The variations in these various factors in renal disease

\* *Journ. of Biol. Chem.*, xvii., p. 305.

have been investigated by Palmer and Henderson,\* who find that in certain types of the disease ("High Ratio") there is a remarkable increase in R, mainly due to a deficit in the excretion of ammonia. In other types of renal disease the various factors are nearer to normal, as can be seen from the table below.

| $P_H$ | Volume | A   | N   | A+N | R    | Type         |
|-------|--------|-----|-----|-----|------|--------------|
| 5.2   | 1650   | 318 | 165 | 483 | 2.08 | High Ratio   |
| 5.2   | 1086   | 287 | 276 | 563 | 1.03 | Medium Ratio |
| 5.6   | 1187   | 244 | 341 | 585 | 0.72 | Low Ratio    |

It would seem that an important factor in renal disease is a difficulty in the excretion of ammonium salts, so that to maintain the normal reaction of the blood the kidney is forced to excrete an abnormally acid urine. This, in its turn, may cause a further degeneration of the renal tissues.

A further point of interest in connexion with the acidity of the urine is that, according to van Slyke, the alkali reserve of the body can be determined and the condition of "acidosis" diagnosed from the indication given by the analyses described below.†

The method of determining the  $P_H$  of urine is given on p. 29.

### 322. The estimation of titratable acid in urine (Cole's method).

*Principle.* The urine is titrated to  $P_H = 7.45$  (the average reaction of normal blood), using a large form of Cole and Onslow's

\* *Journ. of Biol. Chem.*, xxi., p. 37.

† A full account will be found in the following papers:—Fitz and van Slyke, *Journ. of Biol. Chem.*, xxx., p. 389. Van Slyke, *ibid.* xxxiii., p. 271.

comparator (see p. 21). The concentrations of the indicator in the urine and the two buffer solutions, and also the intensity of the pigment in the three tubes containing urine are kept constant by the addition of equivalent quantities of water and soda respectively.

### Solutions and Apparatus required.

1. A comparator for holding the tubes (see fig. 37). This should be fitted with a ground glass screen, as shewn in the diagram.

2. Tubes of clear resistance glass ( $7 \times 1$  in.) to fit the comparator. These should have the same internal diameter. They can be calibrated by measuring 25 cc. of water from a pipette into a number and selecting those tubes in which the fluid reaches the same level. A better method is to use a hard

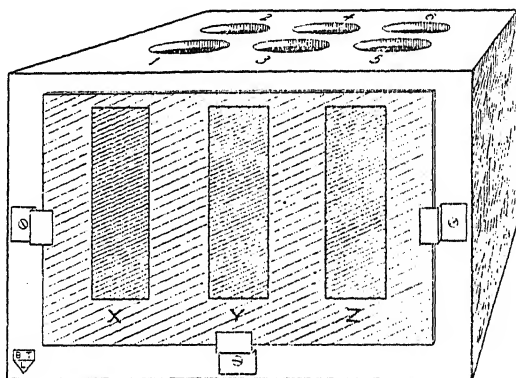


Fig. 37. Cole and Onslow's Comparator for large tubes.

wood gauge, which is slightly conical and is marked with rings corresponding to every  $\frac{1}{64}$  in. diameter. The gauge is pressed into the tubes and those selected of the same internal diameter. The method of using this is shewn in fig. 38. It is convenient to choose sets of tubes and to mark each member of a set with a distinguishing letter by means of a diamond.



Fig. 38.  
Gauge.

3. *Buffer solution*,  $P_H = 7.42$ . Prepared by treating 50 cc. of 0.2 M.  $KH_2PO_4$  with the equivalent of 39.9 cc. of 0.2 N. NaOH and diluting to make 200 cc. with distilled water (see p. 27).

4. *Buffer solution*,  $P_H = 7.47$ . Prepared as above, but use the equivalent of 40.2 cc. of 0.2 N. NaOH.

5. *Standard alkali*, see p. 380. It is convenient to use 0.1 N. from an ordinary burette or 0.2 N. from a microburette.



**Method.**

Measure 20 cc. of the urine into tubes (2), (3) and (6).

Measure 20 cc. of the buffer  $P_H = 7.42$  into (1).

Measure 20 cc. of the buffer  $P_H = 7.47$  into (5).

Place about 20 cc. of water into (4).

Add 10 to 15 drops of 0.02 per cent. phenol red to (1), (3) and (5), using a dropping pipette (fig. 5) and adding exactly the same amount to each tube.

Mix the contents of the tubes by smartly rotating them between the palms of the hands.

Titrate (3) with the standard soda. A precipitate of earthy phosphates may appear and the colour as seen through Y gradually approaches that seen through X. Let the amount of soda added be (a) cc.

To (1) and (5) add (a) cc. of distilled water from a burette or pipette.

To (2) and (6) add (a) cc. of the standard soda. Read the burette.

Complete the titration of (3) until the colour as seen through Y is intermediate between that seen through X and Z. The tubes must be spun just before the observation is made to ensure an equal distribution of any precipitate. Let the amount of soda required for this operation be (b) cc.

*Calculation.*

20 cc. of urine require (a) + (b) cc., say (A) cc. of the soda, which is (c) times Normal.

So 100 cc. require  $(A) \times 5$  cc. of the soda.

So 100 cc. contain  $(A) \times 5 \times 10 \times (c)$  cc. of 0.1 N. acid.

NOTES.—1. It is important that the examination be made in fresh urine. Should this be impossible a little toluol should be added to the specimen to prevent the ammoniacal fermentation of the urea.

2. Should the urine be so concentrated that a precipitate of urates separates out, the urine may be diluted with an equal volume of distilled water and the mixture gently warmed till it clears. It is then cooled under the tap and the estimation made as described above, allowance being made for the dilution in the final calculation.

3. By a similar method, enzyme solutions, digestion mixtures, etc., can be brought to any desired hydrogen-ion concentration. Suitable buffer solutions and indicators can be prepared according to the directions given in pages 22 to 28.

### C. The Pigments of Urine.

**Urochrome** is the chief pigment of normal urine. It is a yellow substance which has no definite absorption band. Nothing certain is known as to its constitution or origin, except that it is apparently not derived from the bile pigments. It has marked reducing properties.

**Urobilin** occurs in fresh normal urine as its chromogen, urobilinogen. This is converted into urobilin by acids or by the action of light and oxygen. The amount excreted is markedly increased in fevers, in diseases of the liver and bile passages, by destruction of the red corpuscles, especially in pernicious anaemia and malaria, and during the absorption of blood clots. In certain of these cases the urobilin itself is found in the urine, and can be identified by its characteristic absorption band, urobilinogen not giving a definite band.

Urobilinogen is a pyrrol body and is responsible for Ehrlich's reaction with *p*-dimethyl-amino-benzaldehyde.

The origin of urobilin from the bile pigments is discussed on page 268. It may be added that the urobilin absorbed from the bowel into the circulation is mostly excreted by the liver into the bile, so that only a small portion reaches the urine. Should the liver cells be injured, or should there be any interference with the circulation through the liver, there is a considerable increase in the excretion of either urobilin or urobilinogen in the urine.

If the common bile duct is completely occluded by a gall stone or by a growth the urobilin and urobilinogen are absent from the urine and faeces. Should the obstruction be removed there is often a period during which the amounts of these substances in the urine is exceptionally large.

**Uroerythrin** is found in small amounts in normal urine. It is increased in fever and certain diseases of the liver.

It is soluble in amyl alcohol. Solutions have a reddish colour, but are unstable to light.

The pigment is usually associated with the urates or uric acid of the urine.

**Haematoporphyrin** is found in traces in normal urine. There is a certain increase in fevers, and some other diseases, but a very marked increase in certain cases of poisoning by sulphonal or trional, especially in women.

**Urorosein** occurs in urine as a chromogen which is converted into the pigment by the action of strong acids, such as hydrochloric and sulphuric.

It is insoluble in ether and is thus distinguished from indigo blue formed in the test for indican. (Ex. 318.)

The chromogen seems to be an indol body, possibly indol-acetic acid.

323. Note the colour of normal urine and examine some in a beaker by the spectroscope. Note that there are no definite absorption bands, but a general absorption of the violet. Urochrome, the chief urinary pigment, yields no bands.

324. Saturate at least 200 cc. of urine with ammonium sulphate. Filter off the precipitate and let it dry completely in the air. Extract it with a small amount of strong alcohol. A brownish solution containing urobilinogen is obtained. Treat this with a few drops of hydrochloric acid: the urobilinogen is converted to **urobilin**. Examine with the spectroscope, and note a single absorption band situated at the junction of the blue and the green. Its centre is about  $\lambda$  490.

325. **Bogomolow's test for urobilin or urobilinogen.** Treat 10 cc. of the urine with 10 drops of 20 per cent. copper sulphate. Add about 4 cc. of chloroform, place the thumb on top of the tube and invert 10 times without shaking. If abnormal amounts of urobilin or urobilinogen are present, the chloroform layer is coloured yellow.

Place the finger on the upper end of a dry 5 cc. pipette and insert the lower end into the chloroform layer. Suck up the chloro-

form solution and transfer it to a dry tube. The chloroform usually separates as a clear fluid, which is of a faint pink colour if urobilin is present. A characteristic absorption band, with centre about  $\lambda$  500 can be seen.

326. **Schlesinger's test for urobilin.** To 10 cc. of urine add 3 drops of a 5 per cent. alcoholic solution of iodine (to convert urobilinogen to urobilin). Into another test-tube place 1 gram. of zinc acetate and 10 cc. of absolute alcohol. Mix the two solutions and repeatedly decant until all the zinc acetate has dissolved. Filter. Examine the filtrate in a test-tube, 16 mm. wide, in daylight falling from behind the observer. A green fluorescence is seen if urobilin or urobilinogen are present.

NOTE.—The above method is a modification introduced by Marcussen and Hansen (*Journ. Biol. Chem.*, xxxvi., p. 381). They state that ammoniacal urines should be acidified with acetic acid. They find that in patients suffering from liver complaints they can detect the fluorescence when the urine has been diluted 40 to 80 times, and are of the opinion that unless it can be detected in a dilution of 1 in 20 a pathological urobilinuria has not been definitely established.

#### D. The Inorganic Constituents.

##### *Kations.*

**Sodium and potassium** are found to the extent of 3.2 gram.  $K_2O$  and 5.23 gram.  $Na_2O$  per diem. The ratio  $K_2O : Na_2O$  generally equals 1 : 1.54.

During starvation this can rise as high as 3 : 1, owing to the excretion of the potassium of the tissues, sodium being found in a much smaller amount than potassium. The same is found in all wasting diseases.

**Calcium and Magnesium** are mainly excreted by the bowel. The amounts in urine are 0.17 to 0.62 gram.  $CaO$  and 0.19 to 0.31 gram. of  $MgO$ .

The amounts of these alkaline earths in the urine are increased by the administration of organic acids, or in conditions such as diabetes in which the formation of such acids is increased.

**Iron** also is mainly excreted by the bowel. It is found in human urine only in organic combination, and then only to the extent of 0.5 to 10 milligrams per diem.

*Anions.*

**Chlorides** form the chief part of the anions of the urine. The amount excreted is often calculated as if it all existed as NaCl, though the amount of sodium in the urine is normally not sufficient to combine with all the chlorine. The amount in the urine depends largely on the amount in the food, but since an important function of the kidney is to maintain a constant osmotic pressure of the tissue fluids, mainly by variations in the amount of NaCl excreted, it follows that anything tending to cause a change in the osmotic equilibrium in the body is liable to alter the excretion of chlorides in the urine.

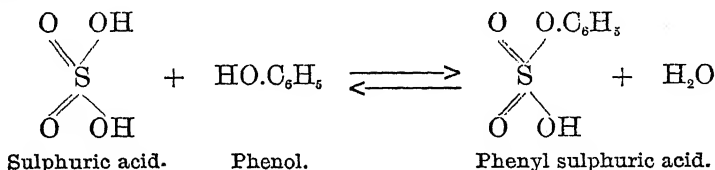
Thus during starvation and during the formation of exudates in pneumonia the chlorides may disappear from urine. The amount of Cl excreted per diem is about 7 grams. Reckoned as NaCl it is 12 grams.

For the method of estimation see p. 355.

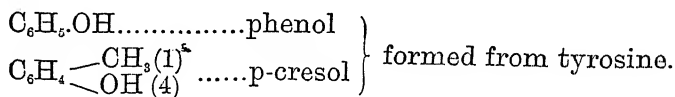
**Sulphates.** Only a small portion of the sulphate excreted in the urine is taken in as such with the food. The greater portion is derived from the oxidation of sulphur containing substances, chiefly proteins. The amount of sulphates is thus a rough measure of the total amount of protein metabolised, the ratio  $\frac{N}{SO_3}$  being usually  $\frac{5}{1}$ .

Sulphates are excreted very rapidly after a protein meal, reaching a maximum about the third hour. This seems to indicate that cystine, the sulphur complex of proteins, is split off and absorbed very early in the digestion of proteins.

**Ethereal Sulphates** are esters formed by the union of sulphuric acid with phenols.



The proportion of the sulphur that is present as ethereal sulphate varies considerably. Folin has shewn that in starvation and on diets relatively deficient in proteins the proportion increases, as does that of the "neutral" sulphur. There is also a marked increase after the administration of certain phenolic substances, or when such compounds are formed in the body by bacterial decomposition, as in intestinal obstruction and severe constipation. In such cases the phenols found conjugated with sulphuric acid are



$\text{C}_8\text{H}_6\text{N.OH} \dots\dots\dots$  indoxyl, formed from tryptophane.

These bodies are poisonous. They unite with sulphuric acid, probably in the liver, to form the innocuous ethereal sulphates.

The ethereal sulphates form soluble barium and benzidine salts, and can be separated from the inorganic sulphates by treatment with barium chloride or benzidine hydrochloride and filtering. They are hydrolysed to the phenol and sulphuric acid by boiling with hydrochloric acid.

**"Neutral" Sulphur.** In urine there is always present a certain amount of sulphur in a form less oxidised than that of a sulphate. The exact nature of the compounds in urine containing sulphur in this form is not yet clear.

It is probable that the amount of "neutral" sulphur in the urine is independent of the total amount of sulphur excreted. It probably varies with the amount of tissue protein metabolised, so that its determination is often of considerable interest.

For the percentages of sulphur excreted in the three forms under different metabolic conditions see page 270.

For the methods of determination of the sulphur see pages 358—361.

**Phosphates.** The phosphates of the urine are present on the one hand as salts of the alkali metals and of

ammonium ; on the other, as salts of the alkaline earths, calcium and magnesium. About 3.9 grams. of  $P_2O_5$  are excreted per diem in the urine. Phosphoric acid forms three series of salts. The formulæ for the three sodium and calcium salts respectively are

Normal phosphate,  $Na_3PO_4$  :  $Ca_3(PO_4)_2$ .

Mono-hydrogen phosphate,  $Na_2HPO_4$  :  $CaH(PO_4)$ .

Di-hydrogen phosphate,  $NaH_2PO_4$  :  $CaH_4(PO_4)_2$ .

The three sodium salts and  $CaH_4(PO_4)_2$  are soluble in water : the other two calcium salts are insoluble. The normal and mono-hydrogen phosphates are alkaline in reaction to litmus : the di-hydrogen phosphates are acid.

The phosphates of the urine are derived partly from the inorganic phosphates of the food, partly from the oxidation of phosphorus-containing substances of the food and tissues, such as nucleo-proteins, lecithins and phospho-proteins, and partly also from the phosphates of bone. The exact share played by these various compounds in forming the urinary phosphates is difficult to determine owing to the fact that a proportion of the phosphates, varying between 12 and 50 per cent., are excreted by the bowel. In this connection it may be noted that alkaline phosphates of the food are more likely to be excreted in the urine than are earthy phosphates.

The excretion of varying amounts of phosphates by the kidney is one of the methods by means of which the reaction of the body fluids is maintained in equilibrium. An increased excretion is always seen in cases of acid poisoning and in the acidosis associated with diabetes.

As soon as the urine shews a certain grade of alkalinity, precipitation of earthy phosphates takes place. This is sometimes known as phosphaturia, but it is not necessarily associated with an increase of phosphates in the urine. In the phosphaturia of juveniles it is probable that there is an excessive amount of calcium in the urine, due to a defective excretion of the large intestine.

A certain amount of phosphorus is found in the urine in an organic form, not as a phosphate. It may be present as glycerophosphoric acid. The average daily amount is about 50 mgms.

For method of estimation see Ex. 414.

327. Test for **chlorides** by adding to about 3 cc. of urine a few drops of pure nitric acid and 3 cc. of a 3 per cent. solution of silver nitrate. An abundant curdy precipitate of silver chloride appears at once. If the chlorides are less in quantity, the solution merely becomes milky or opalescent.

NOTE.—If nitric acid is not added, urates might be precipitated by silver nitrate, especially if the urine be ammoniacal.

328. To a test-tube nearly full of urine add a little strong ammonia and boil. A white flaky precipitate of the **phosphates of calcium and magnesium** is formed. Filter off the precipitate, wash with water, and dissolve in 5 cc. of dilute acetic acid. Divide the solution into two parts. To one part add a solution of potassium oxalate. A white precipitate is produced, showing the presence of **calcium** in the urine.

329. To the other portion of the solution add an equal bulk of strong nitric acid and about 5 cc. of ammonium molybdate. Boil: a yellow crystalline precipitate is produced, showing the presence of **phosphates**.

NOTE.—Neutral urine is very apt to yield a precipitate of earthy phosphates on boiling, owing to the change of reaction due to the evolution of  $\text{CO}_2$  (see notes to Ex. 28).

330. To demonstrate the presence of acid-phosphates in urine. Treat 5 cc. of urine with an equal volume of 5 per cent. solution of barium chloride. Filter repeatedly through a small filter paper till the filtrate is clear. Treat the filtrate with a little baryta mixture and boil. Filter; dissolve the precipitate in nitric acid and boil the solution obtained with ammonium molybdate. The yellow precipitate shows the presence in the urine of acid phosphates, such as  $\text{NaH}_2\text{PO}_4$ .



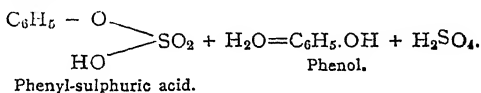
NOTE.—Any alkaline phosphate,  $\text{Na}_2\text{HPO}_4$ , present in the urine is precipitated by  $\text{BaCl}_2$  as  $\text{BaHPO}_4$ . The acid phosphates remain in solution as  $\text{Ba}(\text{H}_2\text{PO}_4)_2$ . On the addition of the alkaline baryta mixture, the acid phosphate is converted into the insoluble alkaline phosphates of barium. If no precipitate is produced when the baryta-mixture is added, there are no acid phosphates present in the sample of urine.

Since the acidity of a sample of urine varies almost directly with the amount of acid phosphates present, as determined by the above method, it is generally held that the acidity of urine is mainly due to the presence of these acid phosphates.

331. Treat 10 cc. of urine with a few drops of strong hydrochloric acid, and about 3 cc. of a solution of barium chloride. A precipitate of barium sulphate is produced as an opaque milkiness. If the precipitate is thick the sulphates are in excess. (The hydrochloric acid is added to prevent the precipitation of phosphates.)

332. To demonstrate the presence of ethereal sulphates. To urine add an equal bulk of baryta mixture (two parts of baryta water to one part of a 10 per cent. solution of barium nitrate). A precipitate is formed consisting of the phosphates and the ordinary *inorganic sulphates*. Filter till quite clear. To the filtrate add a third of its volume of strong hydrochloric acid, boil in a beaker for five minutes, and allow to stand. A faint white cloud of barium sulphate is formed, indicating the presence of *ethereal sulphates* in the urine.

NOTES.—1. The ethereal sulphates form soluble barium salts, but are hydrolysed to sulphuric acid by heating with an acid.



The sulphuric acid thus formed is converted into barium sulphate by the excess of barium present.

2. The solution becomes very dark in colour on boiling with the strong acid, owing to the action of the latter on the aromatic chromogenic substances in the urine.

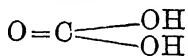
3. Ethereal sulphates can be prepared as follows: warm 10 drops of absolute alcohol with 5 drops of concentrated sulphuric acid in a test-tube. Cool and make alkaline with 5 per cent. soda. Add 10 per cent. barium chloride as long as a precipitate continues to be formed. Boil and filter. The filtrate contains barium ethyl sulphate. Add one-half volume of concentrated hydrochloric acid and boil. A precipitate of barium sulphate is formed.

### E. Urea.

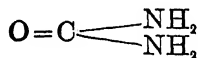
Urea is the compound in which the greater part of the nitrogen is normally excreted in man. The percentage of the urinary nitrogen in the form of urea varies. Normally it is about 86 per cent., but in starvation, or on a diet deficient in proteins, it is only about 60 per cent. It is also low in cases of diabetes accompanied by acidosis (owing to the relatively high percentage of ammonia), and also in certain cases of hepatic disorder, notably acute yellow atrophy of the liver, owing to the non-formation of urea by the disordered liver, its seat of formation in the body.

The total amount excreted per diem by a normal man on an average diet containing 100 grams. of protein is 30 grams.

Urea is also known as carbamide, since it is the diamide of carbonic acid.



Carbonic acid.



Urea.

Urea crystallises in water-free, colourless, long needles, or in four-sided prisms of the rhombic system, which melt and decompose at  $130^\circ - 132^\circ \text{C}$ .

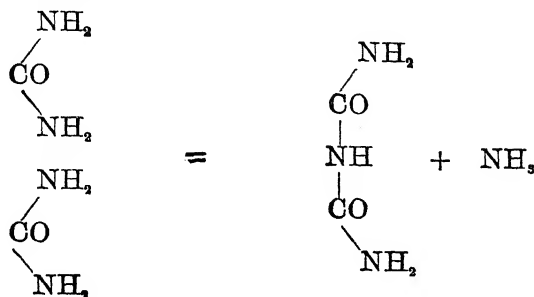
It is soluble in all proportions in hot water, and to the extent 1 : 1 in cold water. In cold alcohol it is soluble to the extent of 1 : 5. It is also soluble in acetone. Insoluble in pure ether and chloroform. The solutions are neutral in reaction.

It forms crystalline compounds with acids. The two most important are urea nitrate  $\text{CH}_4\text{N}_2\text{O} \cdot \text{HNO}_3$ , insoluble in strong nitric acid, and urea oxalate  $(\text{CH}_4\text{N}_2\text{O})_2 \cdot \text{C}_2\text{H}_2\text{O}_4$ , insoluble in oxalic acid.

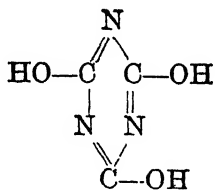
It forms compounds with the salts of the heavy metals, especially with mercuric nitrate (see below, Ex. 341).

With reducing sugars relatively stable compounds are formed, called ureides. They are of importance in connection with the estimation of urea in diabetic urine.

On heating dry urea to  $140^{\circ}\text{C}$ ., ammonia is evolved and biuret formed.



On heating beyond  $140^{\circ}\text{C}$ ., cyanuric acid and ammonia are formed. Cyanuric acid is  $\text{C}_3\text{H}_3\text{N}_3\text{O}_3$ .

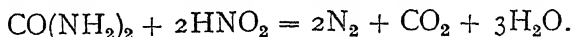


Solutions of urea are decomposed by boiling alkalies into  $\text{CO}_2$  and  $\text{NH}_3$ . They are also similarly decomposed by heating for several hours at  $150^{\circ}\text{C}$ . with acids. This decomposition is readily effected by the addition of magnesium chloride, zinc sulphate or potassium acetate to the solution for the purpose of raising the boiling point.

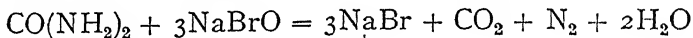
Bacteria, as *micrococcus ureae*, decompose urea into  $\text{CO}_2$  and  $\text{NH}_3$ . This accounts for normal urine rapidly becoming ammoniacal on standing in the air.

Urea is decomposed by the enzyme urease into ammonium carbonate. The enzyme is found in the Soya bean and in other plants. It does not act on any other compound, not even on the substituted ureas. It is therefore used both for the detection (Ex. 343) and also for the estimation (Ex. 401) of urea.

Nitrous acid decomposes urea as follows:—

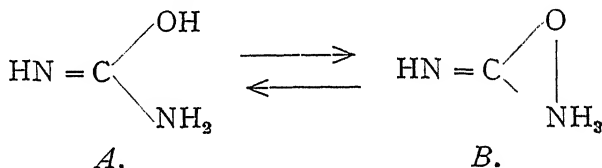


Hypobromites effect a similar decomposition.



Sodium  
hypobromite.

According to Werner\* the reactions of urea are better understood if it be supposed that it exists in two tautomeric modifications, the equilibrium between them depending on the reaction



The *A* form exists in strongly acid solutions. It is decomposed by nitrous acid, like all compounds with the  $\text{NH}_2$  group. The *B* form exists in neutral or alkaline solutions and is not decomposed by nitrous acid. For the convincing evidence on which this view is based the original papers should be consulted.

333. To a watch-glass half full of distilled water add as much solid urea as will lie on a sixpenny-piece. Note the solubility of urea in water.

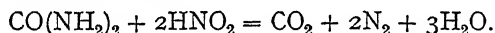
334. Place a drop of the urea solution on a slide, add a single drop of a saturated solution of oxalic acid, mix by stirring with a needle or fine glass rod, cover with a slip and examine the crystals of *oxalate of urea* that separate out. They vary considerably, containing long, thin, flat crystals, often in bundles and rhombic prisms. Draw the crystals.

335. Dilute the urea solution with twice its volume of water. Place a drop on a slide, add a drop of pure nitric acid, cover with a slip, and examine the crystals of *urea nitrate* that separate out. They form octahedral, lozenge-shaped, or hexagonal plates, often striated and imbricated. Draw the crystals.

336. Powder two or three crystals of urea in a watch-glass: rub with a small amount of acetone and warm gently on a water bath. The urea dissolves. Allow most of the acetone to evaporate away, and then place a drop of the remaining solution on a watch-glass. Urea crystallises out as the acetone passes off. Draw the crystals.

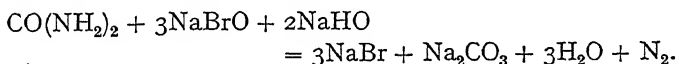
337. Repeat the above exercise, using strong alcohol instead of acetone. Draw the crystals of urea, which are usually very irregular.

338. Dilute the remainder of the aqueous solution left from Ex. 335 with an equal quantity of water, and to a portion of this in a test-tube add some yellow nitric acid (or nitric acid to which a little potassium nitrite has been added). An effervescence and evolution of gas take place.

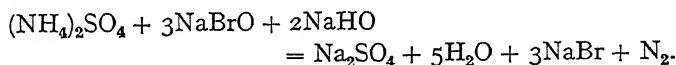


NOTE.—All compounds containing the amino group ( $\text{NH}_2$ ) react in a similar manner when treated with nitrous acid (see Ex. 81). The decomposition of urea with nitric acid is relatively very slow.

339. To another portion of the solution add sodium hypobromite. A marked effervescence and evolution of gas take place.



340. To a few cc. of saturated ammonium sulphate add sodium hypobromite. A marked effervescence and evolution of gas take place.



NOTES.—I. All ammonium salts and all compounds with the amino group give off nitrogen when treated with an alkaline solution of sodium hypobromite.

2. The sodium hypobromite is prepared as follows: dissolve 100 grams. of caustic soda in 250 cc. of water. Cool, and slowly add 25 cc. of bromine, cooling under the tap as the bromine is added. The reaction is as follows:



It must be freshly prepared before use as it undergoes the following decomposition:



3. As a test for urea the reaction with hypobromite is only useful in a negative sense; that is to say, if an effervescence is not obtained urea is absent, but if an effervescence is obtained it does not necessarily follow that urea is present.

341. To some of the urea solution add a solution of mercuric nitrate. A white precipitate of mercuric oxide combined with urea and mercuric nitrate takes place. To the mixture thus obtained add a saturated solution of sodium chloride, drop by drop. The precipitate dissolves, to reappear on a further addition of mercuric nitrate.

NOTES.—1. The precipitate consists of urea and mercuric nitrate and one, two or three molecules of mercuric oxide, depending on the concentration of the two solutions.

2. The solubility in NaCl is due to the formation of mercuric chloride, which is only very feebly ionised in neutral solutions.

342. Treat a solution of urea with Millon's reagent, and heat. A white precipitate is formed, owing to the presence of mercuric nitrate in the reagent. There is also an evolution of gas due to the action of the nitrous acid on the urea.

343. **Specific urease test for urea.** To 4 or 5 cc. of a dilute solution of urea add 4 or 5 drops of phenol red. The colour obtained is generally slightly pinkish. Add traces of very dilute acetic acid by means of a glass rod until the reaction is very faintly acid to the indicator. Warm to about 45° C. Add a large "knife point" of finely ground Soya bean meal, shake and keep the solution warm. The colour changes to a reddish purple, owing to the enzyme converting neutral urea to alkaline ammonium carbonate.

NOTES.—1. In applying the test it is important to see that the reaction is only faintly acid to the indicator. For if a considerable amount of acid and only a small amount of urea be present, the amount of ammonium carbonate formed may not be sufficient to bring the reaction to the point where a pink colour is given with the indicator.

2. Proteins only interfere by acting as buffers. It is not usually necessary to remove them.

3. The test will not succeed in the presence of the salts of the heavy metals, which inhibit the action of the enzyme. A high concentration of buffer salts, such as phosphates or acetates, decreases the delicacy of the test, by preventing considerable changes in hydrogen-ion concentration.

344. To about 4 cc. of a 1 per cent. solution of urea add about 2 cc. of strong soda, mix and divide into two portions, A and B. Boil B for 3 to 5 minutes, adding a little water from time to time to replace that lost by evaporation. Cool under the tap. Add phenol red to each and neutralise by the addition of hydrochloric acid, using concentrated acid at first and finish by dilute hydrochloric. Apply the urease test as described in the previous exercise to the two solutions. A gives a strong test, whilst B gives none, or only a slight one, owing to the destruction of the urea by boiling alkali.

345. Place a little urea in a dry test-tube. Heat carefully over a flame, keeping the upper part of the tube cool. The urea melts and evolves ammonia, whilst a white sublimate condenses on the cooler parts of the tube. Cool the tube, add a little water and shake. Pour the solution into another tube and treat it with an equal bulk of sodium hydroxide and a drop of copper sulphate. A pink colour is produced, due to the biuret formed from the urea.

346. Repeat the experiment, but heat more strongly till the melt solidifies and becomes opaque. Cool, add two or three cc. of water, boil and filter whilst still hot. Divide the solution into two portions, A and B. To A add a few drops of a solution of barium chloride and a single drop of diluted ammonia. A white mass of barium cyanurate is formed on cooling.

To B add some ammoniacal copper sulphate solution and boil. On cooling an amethyst precipitate of copper ammonium cyanurate is deposited.

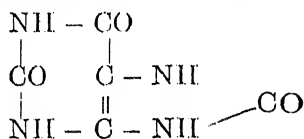
NOTE.—Preparation of ammoniacal copper sulphate. 1 per cent. copper sulphate is treated with *very* dilute ammonia till the precipitate that first forms just redissolves.

347. **Isolation of urea from urine.** Evaporate about 30 cc. of urine to complete dryness, finishing the evaporation on the water bath (to prevent the destruction of the urea). Turn out the flame and rub the residue with about 10 cc. of acetone till it is boiling. Allow the acetone to boil, stirring all the time, till about half of it

has evaporated away. Pour off the acetone into a dry watch glass and allow it to cool. Crystals of urea separate out as silky needles. Demonstrate that they are urea crystals by evaporating to dryness, taking up in a small amount of water and applying the urease test (Ex. 343).

### F. Uric Acid.

Uric Acid,  $C_5H_4N_4O_3$ , is 2-6-8-tri-oxy-purine.



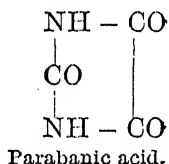
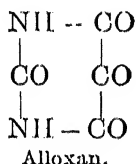
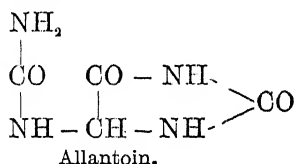
Its relationship to certain of the other purines is indicated on page 63.

When pure it crystallises in microscopic rhombic plates, but when impure it assumes a variety of forms, such as whetstones, dumb-bells, sheaves, rosettes, butchers' trays, etc.

It dissolves to the extent of 1 part in 16,000 parts of cold water and 1,600 parts of hot water. It dissolves in alkalis, and the alkali salts of carbonic, phosphoric, boric, lactic and acetic acids, but not in the ammonium salts of these acids. It dissolves in warm concentrated sulphuric acid to form a sulphate, which is decomposed by the addition of water.

It is precipitated by phosphotungstic acid in the presence of hydrochloric acid, slowly by lead acetate, and completely by picric acid, mercuric chloride and ammoniacal silver nitrate.

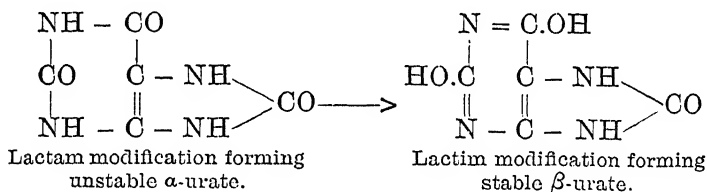
By oxidation, allantoin, alloxan, parabanic acid and urea are formed, depending on the reaction and the reagent employed.





Although the aqueous solutions of uric acid react neutral, it behaves like a disbasic acid  $C_5H_2N_4O_3.H_2$  and can form two series of salts,  $C_5H_2N_4O_3.Na_2$  (neutral, normal, or di-sodium urate) and  $C_5H_2N_4O_3.HNa$  (biurate, acid urate or mono-sodium urate). It is also possible that there is a third form of salt,  $C_5H_2N_4O_3.HNa.C_5H_4N_4O_3$  (quadriurate or hemi-sodium urate), though this may be merely a mixture of its two constituents. The di-sodium salts are more soluble than the mono-sodium, but are only stable in markedly alkaline solutions. In the blood and urine urates exist as mono-sodium salts, which react neutral.

It is interesting to note that there are two modifications of the mono-sodium salt, called the  $\alpha$ - and  $\beta$ -form. The  $\alpha$ -form is more soluble than the  $\beta$ -form, but is unstable, and slowly passes over into the other form. They are probably the salts of the two tautomeric modifications of uric acid described by Fischer :



It is of great interest to observe that in gout the amount of urate in solution in the blood is in excess of the amount of the  $\beta$ -urate that can be held by normal blood. So that in gout it must be present at least, partly, in the unstable  $\alpha$ -form. The deposition of urates in the tissues during an acute attack may be due to the conversion of the unstable  $\alpha$ - into the stable, less soluble  $\beta$ -modification.

Urates are completely precipitated as amorphous ammonium urate by saturation with ammonium chloride.

They exert a reducing reaction on Fehling's solution and towards alkaline silver solutions, this being the basis of Schiff's test.

They yield a characteristic colour reaction when evaporated with nitric acid, the so-called murexide test.

Uric acid occurs to the extent of about 0.7 gram. in the 24 hours' urine, but the amount excreted varies with the diet and the individual.

From its close chemical relationship to the purine bases formed by the hydrolysis of the nucleins of the food and tissues (see p. 63), the view is commonly held that uric acid has its origin in the cellular organs of the body from the oxidation of such substances. Thus we can have uric acid arising exogenously from the free or combined purines of the food and also endogenously from those of the tissues. This view is apparently supported by the fact that the administration of foods rich in nucleoproteins, as sweetbreads, or of certain of the pure purine bases, does cause an increased excretion of uric acid.

It is possible that a certain proportion of the uric acid formed in the body is destroyed by the liver, so that the amount excreted is a balance between that formed and that destroyed.

In gout, in which there is a deposition of uric acid in the tissues, the excretion is decreased before an acute attack, is increased during the attack, and then falls again. In this condition there is a recognisable amount of uric acid in the blood (see above). For the method of estimation in urine see p. 343.

348. Treat a small amount of uric acid with 10 cc. of 2 per cent. sodium carbonate. Heat nearly to boiling and cool. Note that a considerable portion of the uric acid has dissolved in the form of a urate.

349. Filter the solution and treat a portion with a drop or two of strong hydrochloric acid and shake. A white crystalline precipitate of uric acid separates out, showing that uric acid is very insoluble in water. Allow the crystals to settle, remove a few by means of a pipette, and examine them microscopically. They usually form rhombic plates. Draw the crystals.

NOTE.—If the solution is very strong, the uric acid may separate out in an amorphous form. Should this be the case, make the solution alkaline and heat to dissolve. Whilst still hot add some HCl and allow the tube to cool slowly.

Uric acid can assume a great variety of crystalline forms, resembling dumb-bells, whetstones, butcher-trays, stars, and sheaves.

350. To another portion of the solution add two drops of ammonia and saturate with ammonium chloride. A white amorphous precipitate of ammonium urate is formed.

NOTE.—This is the basis of Hopkins' original method for the estimation of urates in urine. It is an important reaction for separating urates from physiological fluids, such as urine (see Ex. 359), since no other organic substance, likely to be met with in physiological analysis, is precipitated by saturation with ammonium chloride. The murexide reaction can be applied to the precipitate obtained.

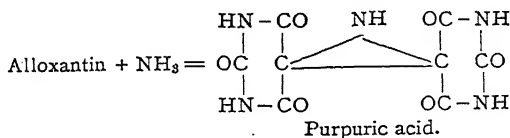
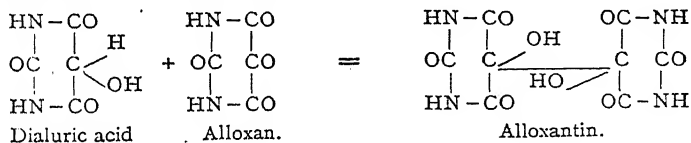
351. Treat a little uric acid with a little strong sulphuric acid: it dissolves. Pour the solution into water: the uric acid may separate out.

352. **Murexide test.** Treat a little uric acid in a porcelain dish with two or three drops of strong nitric acid. Heat on the water bath till every trace of nitric acid and water has been removed. A reddish deposit remains. Treat this with a dilute solution of ammonia (five drops of ammonia to about a test-tube full of water). The residue turns reddish-violet in colour. Add a little caustic soda. The colour turns to a blue-violet.

NOTES.—1. This important test needs a certain amount of care. The heating must be performed on the water-bath, and should be continued as long as is necessary to ensure the complete removal of every trace of nitric acid.

2. Xanthine and guanine give a yellow substance (nitro-xanthine) when treated with nitric acid. On evaporation the colour goes to a violet shade, which turns yellow with dilute ammonia. Adenine and hypoxanthine give no colour reaction.

3. The chemistry of the reaction is as follows: From uric acid arises by oxidation dialuric acid and alloxan. They condense together to form alloxantin. By the action of ammonia on alloxantin, purpuric acid is formed. Murexide is ammonium purpurate.



353. **Schiff's test.** Treat a very small amount of uric acid with a few cc. of sodium carbonate. Pour the solution on to filter paper moistened with silver nitrate. A black stain of reduced silver immediately results.

NOTE.—This useful test cannot be applied in the presence of chlorides. It is important to note that the uric acid is dissolved in sodium carbonate, not the hydroxide, as the latter gives a precipitate of the brown silver hydroxide, which completely obscures the reduction. An amount of sodium carbonate in excess of that required to dissolve the uric acid must be added, as the reduction only takes place in the alkaline condition.

354. **Folin's test.** To a *very* small pinch of uric acid in a beaker add 20 cc. of a saturated solution of sodium carbonate. Stir till the uric acid has completely dissolved, add 1 cc. of Folin's uric acid reagent. A blue colour is obtained.

NOTES.—1. **Preparation of Folin's solution.** 100 grams. of pure sodium tungstate, 102 cc. of pure ortho-phosphoric acid (B.P. 66.3%) and 750 cc. of distilled water in a flask fitted with a reflux condenser are boiled for 2 hours. On cooling the solution is diluted to 1 litre.

2. The solution also gives a blue colour with polyphenols. It is used for the microchemical estimation of uric acid in urine.

355. Dissolve a little uric acid in sodium carbonate by boiling. Add 5 cc. of Fehling's solution and boil for a considerable time. Note the peculiar reduction of the copper, and compare it with the reduction obtained with glucose.

356. Similarly try the effect of uric acid on Nylander's (Ex. 105) and Benedict's (Ex. 100) solutions. A reduction is not obtained.

357. Dissolve some uric acid in sodium carbonate, add an excess of ammonia and treat with silver nitrate. A white amorphous precipitate of a silver compound of uric acid is formed.

NOTE.—Xanthine, hypoxanthine and other substances in urine closely related to uric acid are similarly precipitated by ammoniacal silver nitrate.

358. **A solution of sodium urate and urea is provided. To prepare crystals of uric acid and of urea.**

Heat a test-tube nearly full of the solution to boiling point and add strong hydrochloric acid till the reaction is distinctly acid. Allow the tube to cool slowly; the uric acid crystals separate out.

Cool thoroughly under the tap. Filter off the uric acid. Neutralise the filtrate with sodium carbonate and evaporate to dryness, finishing the process on the water-bath, to prevent the conversion of the urea to biuret (see Ex. 345). Extract the residue with strong alcohol or acetone. The alcohol or acetone solution is carefully evaporated to dryness, and the urea crystallises out.

**359. To demonstrate the presence of uric acid in urine.**

To 50 cc. of urine add powdered ammonium chloride and stir till the solution is saturated. Add three drops of strong ammonia and stir again. Allow the excess of ammonium chloride to settle for 15 secs. and pour off into another beaker. Note the gelatinous precipitate of ammonium urate. Filter : scrape the precipitate off the paper and transfer it to an evaporating dish. Add three or four drops of strong nitric acid and place the dish on the water-bath till a pink, dry residue is obtained. Treat this with a little dilute ammonia : the purple colour produced indicates the presence of urates in urine (see Exs. 350 and 352).

**360. Folin's method of demonstrating the presence of uric acid in urine.** To 1 to 2 cc. (20 drops) of urine in an evaporating dish add one drop of a saturated solution of oxalic acid and evaporate to *complete* dryness on a water-bath. Allow to cool, add 10 cc. of strong alcohol and allow to stand for five minutes to extract the polyphenols. Carefully pour off the alcohol. To the residue add 10 cc. of water and a drop or two of saturated sodium carbonate. Stir to secure complete solution of the uric acid and transfer to a beaker. Add 1 cc. of Folin's uric acid reagent (Ex. 354) and 20 cc. of saturated sodium carbonate solution. The blue colour that results indicates the presence of uric acid.

**361.** Urine has been treated with about one-fiftieth its bulk of strong hydrochloric acid, and allowed to stand from twelve to twenty-four hours. Note the brown crystals of uric acid that have formed on the sides of the vessel. Examine them microscopically : they form very irregular crystals, usually arranged in sheaves. Draw the crystals.

NOTE.—The chief pigment that associates itself with uric acid and urates is known as uroerythrin (see p. 278).

### G. Purine bases, other than uric acid.

The most important of these found in normal urine are hypoxanthine, xanthine and adenine (see p. 62), derived from the metabolism of food and tissue nucleins: heteroxanthine (7-methyl-xanthine) and paraxanthine (1, 7-dimethyl-xanthine) derived from the breakdown of caffeine (1, 3, 7-trimethyl-xanthine) and theobromine (3, 7-dimethyl-xanthine) of the coffee, tea and cocoa ingested.

In man the methylated xanthines constitute the greater part of these purine bases. But it is interesting to note that the non-methylated ones are much increased in fever. Also during severe muscular exercise there is an increase, accompanied by a decrease of uric acid. After the exercise there is an increase of uric acid, and a decrease of the other purines.

The simplest method of estimation is to determine uric acid nitrogen by the method in Exs. 394-397, and the total purine nitrogen by applying Kjeldahl's method to the total purines precipitated by ammoniacal silver nitrate (Ex. 357). The difference is the nitrogen of the purine bases.

### H. Creatinine and Creatine.

The chemical relationships of these bodies are described on p. 178. In normal human urine creatinine is always present, but creatine only after a meat diet, being derived from that of the food. Creatine, however, is a normal constituent of the urine of children.

Creatinine seems to be a product of tissue metabolism, and the amount excreted is regarded by Folin as a measure of endogenous metabolism. (See tables B and C, p. 270.) There is an increase in complete starvation and in fevers, due to the increased tissue breakdown. E. Mellanby has drawn attention to the fact that the liver is probably the seat of formation of creatinine. Thus in most diseases of the liver there is a decreased excretion, an important

exception being hepatic carcinoma, in which condition the urinary-creatinine is increased and is accompanied by creatine. Creatine is excreted when the muscles of the body are broken down. This explains the presence of creatine in urine during starvation and in fevers.

When creatinine is given by the mouth it is mainly excreted unchanged, but a small portion is broken down into unknown products. When creatine is administered it also is chiefly excreted unchanged, but a certain percentage is destroyed in the body. The amount excreted unchanged is considerably increased with diets rich in proteins.

*Properties.* Creatinine dissolves in 11 parts of water and 102 parts of alcohol at 16° C. It is insoluble in ether. Its solutions are neutral or very slightly alkaline in reaction.

Creatinine is precipitated by phosphotungstic acid, by picric acid, and by the salts of the heavy metals. It forms a characteristic compound with zinc chloride, which is used for the preparation of standard solutions.

Alkalies convert it slowly into creatine. On boiling with barium hydroxide it is converted into urea and sarcosine (see p. 178).

Creatinine reduces Fehling's solution, but not Benedict's or Nylander's solutions.

Creatine is converted to creatinine by heating with acids (see Ex. 226). It can be estimated by making determinations of creatinine before and after heating the urine with acid. If aceto-acetic acid is present Graham has found that both results are liable to considerable error (see Graham and Poulton, *Proc. Roy. Soc.*, LXXXVII., B., p. 205). For the method of estimation see p. 338.

### **Preparation of Creatinine from urine.\***

#### *(i.) Preparation of creatinine picrate.*

It is best to work on 10 litres of urine at least. Dissolve 40 grams. of picric acid in 100 cc. of boiling alcohol and use 18 grams. of picric acid per litre of urine. Pour the hot solution directly into the urine, stirring well during the addition.

\* (Benedict, *Journal of Biological Chemistry*, xviii., p. 183.)

Allow to stand over-night and syphon off the supernatant fluid. Drain the residue on a Buchner funnel and wash with cold saturated picric acid and then drain dry.

(ii.) *Decomposition of the picrate.*

Treat the dry creatinine picrate with concentrated hydrochloric acid in a mortar, using 60 cc. of acid for every 100 grams. of the picrate. Stir thoroughly with the pestle for 5 minutes. Filter by suction, using a hardened filter paper. Wash residue twice with enough water to cover it, sucking dry each time. Transfer the filtrate at once to a large flask and neutralise with solid heavy magnesium oxide. Add it in small amounts at a time, cooling under the tap after each addition. The solution turns light yellow when all the acid has been neutralised. Filter with suction and wash the residue twice with water. At once add a few cc. of glacial acetic acid to the filtrate and pour it into 4 volumes of 95 per cent. alcohol. Allow to stand at least 15 minutes and filter under suction.

(iii.) *Preparation of creatinine zinc chloride* ( $C_4H_7N_3O)_2 \cdot ZnCl_2$ .

Treat filtrate with a 30 per cent. solution of zinc chloride, using 3.5 cc. for each litre of urine taken. Allow to stand over-night in a cool place. Pour off the supernatant fluid and then collect the creatinine zinc chloride in a Buchner. Wash once with water, then thoroughly with 50 per cent. alcohol, then 95 per cent. alcohol and dry. The product should be a nearly white, light crystalline powder.

*Yield:* 1.2 to 1.5 grams. per litre of urine.

(iv.) *Recrystallisation of creatinine zinc chloride.*

10 grams. are treated with 100 cc. of distilled water and then with 60 cc. of N. sulphuric acid. The solution is heated to boiling till a clear solution is obtained. About 4 grams. of pure decolourising charcoal are added and the boiling continued for about 1 minute. The solution is filtered off through a small Buchner, the filtrate being refiltered through the same funnel two or three times till it is quite clear. The residue is washed with a little hot water and the total filtrate transferred to a beaker. It is then treated hot with 3 cc. of a strong solution of zinc chloride and about 7 grams. of potassium acetate dissolved in a little hot water. After 10 minutes the solution is treated with an equal volume of strong alcohol and allowed to stand for some hours in a cool place. The crystals are filtered off and stirred up with about twice their weight of cold water, filtered, washed with a little water, and then with alcohol.

*Yield:* 85 to 90 per cent. of the crude material.

(v.) *Conversion of the zinc chloride compound to creatinine.*

The powdered, recrystallised compound is placed in a dry flask and treated with 7 cc. of concentrated aqueous ammonia for every gram. taken. It is slightly warmed and gently agitated until a clear solution is obtained, care being taken to drive off as little ammonia as possible. The flask is then stoppered and placed in an ice box for an hour or two. Pure creatinine crystallises out.

*Yield:* 60 to 80 per cent. of the theoretical.

362. **Jaffe's test for creatinine.** To 5 cc. of urine add a few drops of a saturated aqueous solution of picric acid and of a 10 per cent. solution of sodium hydroxide. A red colouration is produced owing to the formation of picramic acid (see Ex. 108).



363. **Weyl's test for creatinine.** To 5 cc. of urine add a few drops of a freshly prepared 5 per cent. solution of sodium nitroprusside. Add a 5 per cent. solution of sodium hydroxide, drop by drop. A ruby-red colour appears. Boil. The solution turns yellow. Acidify with strong acetic acid and heat. A green tint appears and a precipitate of Prussian blue may separate.

NOTE.—It is essential to get the ruby-red colour. The formation of Prussian blue is apt to occur with a variety of other substances.

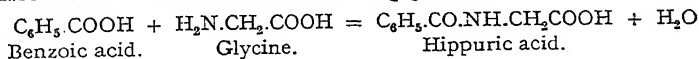
### I. Ammonia.

Ammonia is a constituent of normal urine, being present to the extent of about 0.7 grams. per diem. There is an increased excretion following the administration of ammonium salts of inorganic acids, in certain cases of hepatic disease, and as a result of acid poisoning. This last condition ("acidosis") can be produced by the administration of inorganic acids or by the excessive formation of acids in the body, especially if this is not accompanied by an increased intake of alkalis. Thus it is seen in severe diabetes, in starvation, and in delayed chloroform poisoning, the acids formed being aceto-acetic and  $\beta$ -oxy-butyric acids. In certain forms of renal disease there is a decreased excretion (see p. 275).

For methods of estimation see Exs. 398 to 400.

### J. Hippuric Acid.

Hippuric acid is formed in the kidney by the condensation of benzoic acid with glycine.



The amount excreted by a normal individual on a mixed diet is about 0.7 grams. per diem. It is increased by a vegetable diet, owing to the presence in most plant foods of an aromatic complex that is oxidised to benzoic acid in the body.

Hippuric acid crystallises in 4-sided prisms, somewhat resembling triple phosphate. It melts at  $187.5^\circ \text{C.}$ : above

this temperature the melt becomes red and is decomposed into benzoic acid, benzonitrile and prussic acid. It is soluble in hot water, alcohol and ethyl acetate: insoluble in benzene and petroleum ether: only slightly soluble in cold water, alcohol, ether and chloroform. It forms an insoluble ferric salt. By hot acids or alkalies it is hydrolysed to benzoic acid and glycine. When evaporated with strong nitric acid, nitrobenzene is formed.

364. **Isolation from urine by Roaf's method.** 500 cc. of the urine of a horse or cow are treated with 125 grams. of ammonium sulphate and 7.5 cc. of concentrated sulphuric acid. On standing for 24 hours the hippuric acid crystallises out. Filter off the crystals, and wash with a little cold water. Dissolve in a small amount of hot water, boil with a little adsorbent charcoal, filter, concentrate if necessary, and allow to stand for 24 hours.

365. To a little hippuric acid in a small evaporating dish add 1 to 2 cc. of concentrated nitric acid and evaporate to dryness in a water-bath in the fume chamber. Transfer the residue to a dry test-tube, apply heat, and note the odour of nitrobenzene (artificial oil of bitter almonds).

366. Neutralise a solution of hippuric acid with dilute caustic soda. Add a few drops of ferric chloride. A cream-coloured precipitate of the ferric salt of hippuric acid is formed.

## K. Certain Constituents of Abnormal Urine.

### 1. *Albumin and Globulin.*

"Albuminuria" is the name given to the condition in which a heat-coagulable protein is found in the urine, no matter whether the protein present is albumin or globulin. As a rule both proteins are present, but albumin is generally greatly in excess of the globulin.

Albuminuria can be renal ("true") or accidental ("false"). Renal albuminuria can be brought about by an alteration in the blood pressure in the kidney, by a change in the composition of the blood, or by an alteration in the

structure of the kidney. In accidental albuminuria, the protein is not passed by the kidney, but gains access to it lower down in the urinary tract. It is generally accompanied by haemoglobinuria.

For routine work the author now uses the sulphosalicylic test. It is very rapid and conclusive.

For the method of estimating the albumin see Exs. 420, 421.

367. **Boiling test.** Filter the urine till it is clear. If it will not filter clear, as when infected with bacteria, shake with kieselguhr and filter again. If the urine be alkaline to litmus, make it faintly acid by the cautious addition of 1 per cent. acetic acid. Fill a narrow test-tube three parts full with the clear urine, incline it at an angle and boil the upper layer by means of a very small flame. A turbidity indicates either albumin or earthy phosphates (see note 2 to Ex. 28). Add one or two drops of strong acetic acid, boiling after the addition of each drop. Any remaining turbidity indicates the presence of albumin.

368. **Heller's test.** Place about 3 cc. of pure nitric acid in a narrow test-tube. Float about 3 cc. of filtered urine on the surface of this, using a pipette to avoid mixing. A white ring at the junction of the fluids indicates the presence of albumin.

NOTES.—1. The white ring is due to the formation of metaprotein by the action of the acid on the albumin, and the insolubility of the metaprotein in the strong nitric acid (see Exs. 21 and 40).

2. A coloured ring is usually produced owing to the oxidation of certain urinary chromogens.

3. In very concentrated urine, a white ring of urea nitrate may form. It usually has very sharply defined borders.

4. If the urine is very rich in urates, a precipitate of uric acid may form at the junction of the fluids, or, more commonly, somewhat above the nitric acid. Urea and uric acid are distinguished from albumin by the previous dilution of the urine with two or three volumes of water.

5. The presence of resinous substances in the urine of patients who have been treated with balsams leads to the development of a white ring or cloud that disappears on treatment with alcohol.

6. Urine rich in albumose may give a white cloud that disappears on warming.

7. Urine that has been preserved by the addition of thymol gives a ring of nitrosothymol or nitrothymol. The thymol can be removed by gentle agitation with petroleum ether.

369. **Roberts' test.** Repeat the previous exercise, using Roberts' reagent in place of the nitric acid. A white ring at the junction of the fluids indicates albumin.

NOTES.—1. Roberts' reagent is prepared by adding 1 volume of pure nitric acid to 5 volumes of a saturated solution of magnesium sulphate.

2. Coloured rings are not formed, and so confusion is avoided.

370. **Spiegler's test.** Render the urine faintly acid with acetic acid and repeat the above test, using Spiegler's reagent in place of Roberts'. A white ring indicates the presence of albumin.

NOTES.—1. Spiegler's reagent consists of:

|                   |    |    |            |
|-------------------|----|----|------------|
| Mercuric chloride | .. | .. | 40 grams.  |
| Tartaric acid     | .. | .. | 20 grams.  |
| Glycerine         | .. | .. | 100 grams. |
| Sodium chloride   | .. | .. | 50 grams.  |
| Distilled water   | .. | .. | 1000 cc.   |

2. The reaction is also given by albumoses and peptones.

3. The test serves to show 1 part of albumin in 250,000. It is almost too delicate for ordinary clinical work, as a large number of apparently normal urines give a positive reaction.

371. **Sulphosalicylic test.** To 1 or 2 cc. of the clear, filtered urine add a large "knife point" or a few drops of a 20 per cent. solution of sulphosalicylic acid (see Ex. 18). A cloud or precipitate indicates the presence of albumin.

## 2. *Albumoses.*

Albumoses are found in the urine in certain cases of degeneration of the intestinal epithelium ("alimentary albumosuria"). Also in a variety of other conditions such as in the absorption of pneumonic exudates, in some cases of an increased breakdown of the tissues in certain fevers, in the puerperium and in urine containing semen.

The albumose present seems to be a secondary albumose.

372. Remove any albumin that may be present by heat coagulation. To the filtrate apply Spiegler's test (Ex. 370). A white ring indicates the presence of albumose.

## 3. *Bence-Jones' Protein.*

In certain cases of disease of the bone marrow (multiple myeloma), and possibly in osteomalacia, a protein with

peculiar properties is found in the urine. It is named after Bence-Jones, who first described the condition. It has the property of coagulating at temperatures under  $55^{\circ}\text{C.}$ , of redissolving to a clear solution on boiling and of reappearing on cooling. It is precipitated by half-saturation with ammonium sulphate. It is not precipitated on dialysis.

Hopkins has shewn that the solution of the heat coagulum on boiling depends on the presence of neutral salts, those with divalent cations (as  $\text{CaCl}_2$ ) being most potent in neutral or faintly acid solutions, and those with divalent anions (as  $\text{K}_2\text{SO}_4$ ) in faintly alkaline solutions.

Hopkins has also shewn that the protein excreted is formed in the body, either in the marrow or as a result of the influence of the growth on general metabolism. The amount in the urine is independent of the nature or amount of the proteins of the food. The nitrogen of the protein excreted may be as high as one-third of the total urinary nitrogen.

373. If necessary make the suspected urine faintly acid with acetic acid. Heat carefully by immersing in a beaker of warm water. The urine becomes turbid at  $40^{\circ}$  to  $45^{\circ}\text{C.}$ , and shows a flocculent precipitate at  $60^{\circ}\text{C.}$  On raising the temperature to  $100^{\circ}\text{C.}$  the precipitate partially or completely disappears. On cooling it reappears.

#### 4. *Blood Pigments.*

Blood pigments may occur in pathological urine in intact corpuscles ("haematuria") or free in solution ("haemoglobinuria").

Haematuria can be recognised by determining the presence of red corpuscles by a microscopic examination of the sediment obtained by centrifugalising the urine. It occurs with gross lesions of the kidney or any part of the urinary tract, so that blood passes directly into the urine. If the blood comes from the kidney it is well mixed with the urine. If the blood comes from the bladder

or genital organs it often forms a clot. In haematuria the urine often has a characteristic smoky appearance, and it is always associated with albuminuria. Haemoglobinuria is a result of haemolysis. It therefore follows a variety of infectious diseases, transfusion of blood, the absorption of haemolytic substances, such as many aromatic compounds, severe burns and scalds. Methaemoglobin is nearly always present.

The simplest method of detecting blood is by means of the benzidine test, provided that the necessary reagents are to hand.

374. **Heller's test.** Boil 10 cc. of urine with a little 40 per cent. sodium hydroxide, and allow the tube to stand for a while. A red deposit indicates the presence of blood-pigment in the urine. Pour off the supernatant fluid and acidify with acetic acid. The precipitate dissolves only partially, leaving a red residue.

NOTES.—1. The alkali converts the pigment into haematin, which is precipitated with the earthy phosphates.

2. Certain substances, such as cascara sagrada, rhubarb, senna and santonin cause the urine to give a similar red precipitate when boiled with alkali. But in these cases the precipitate dissolves completely in acetic acid.

375. **Schumm's spectroscopic test.** Treat 50 cc. of the urine with 5 cc. of glacial acetic acid and 50 cc. of ether. Shake thoroughly in a separating funnel. Allow to stand and add a drop or two of alcohol to obtain a separation of the layers. Run off the urinary layer. To the ether add 5 cc. of water, shake and run off the water. To the washed ether add ammonia and shake for half a minute, cooling under the tap. The reaction must be markedly alkaline after shaking. Run off the lower coloured layer into a tube, add 5 to 10 drops of ammonium sulphide solution and examine spectroscopically for the bands of haemochromogen. (Ex. 305.)

376. **Benzidine test.** To a large "knife point" of benzidine in a perfectly clean, dry test-tube add about 3 cc. of glacial acetic acid and agitate for about a minute. Add an equal volume of "10 volumes" hydrogen peroxide. Mix and pour one-half into another clean, dry test-tube. To one of the tubes add 1 cc. of the

suspected urine. The fluid rapidly acquires a deep blue tint if blood pigment is present. Should the untreated fluid also develop a blue tint, the test should be repeated, the control tube being treated with 1 cc. of a normal urine. By following this procedure the test is a very conclusive one. The reaction can be applied to an *acid* ether extract prepared by the method given in the preceding exercise.

### 5. *Bile.*

The constituents of the bile are found in urine when the bile duct is obstructed by a calculus or by catarrh. The bile is absorbed into the lymphatics, passes into the circulation and reaches all parts of the body, the pigments causing a staining of the various tissues. The condition is known as jaundice.

The absence of bile salts from the urine does not exclude the possibility of the presence of bile pigments. With continued obstruction of the bile passages the formation of bile salts seems to decrease. Urine containing bile often has a characteristic appearance.

377. **Cole's test for bile pigments.** Treat 10 to 15 cc. of the urine with 2 drops of saturated magnesium sulphate and proceed as directed in Ex. 318. If a hand centrifuge is available, the test is more sensitive if an excess of barium chloride is added to the unheated urine and the precipitate driven down by spinning in the machine. The supernatant fluid is poured off as cleanly as possible, the precipitate stirred with the alcohol and sulphuric acid, transferred to a test-tube and boiled with the potassium chlorate.

In a certain number of cases the result is obscured by the presence of certain other pigments. In such cases to render the test more delicate, pour off the alcoholic solution from the barium sulphate into a dry tube. Add about one-third its volume of chloroform and mix. To the solution add about an equal volume of water, place the thumb on the tube, invert once or twice and allow the chloroform to separate. It contains the bluish pigment in solution.

378. **Hay's test for bile salts.** Sprinkle the surface of some urine in a test-tube with flowers of sulphur. The particles fall to the bottom of the tube if bile salts are present. (See Ex. 316.)

379. **Oliver's test for bile salts.** Acidify the urine with acetic acid and filter if necessary. To it add a clear 1 per cent. solution of Witte's peptone, also acidified with acetic acid. A white precipitate indicates bile salts. (Ex. 317.)

## 6. Glucose.

Glucose is not, strictly speaking, an abnormal constituent of urine. The author was finally convinced of this some years ago when working at a method for the *detection* of small amounts of glucose in urine\* (see Ex. 381). Folin† confirmed this, using practically the same method. Recently Benedict and Osterberg‡ have introduced a new method for the *estimation* of glucose in normal urine (see Ex. 407), and although it has only been applied to a few individuals, the results obtained are of very great importance, and will probably serve as the starting point for a new attack on the problems of diabetes. According to these observers about 1 gram. of non-nitrogenous reducing substance is excreted per diem, of which about 55 per cent. is not fermentable by yeast, and has not yet been identified. The effect of diet is interesting. The excretion is increased by carbohydrate intake, especially at breakfast. A similar intake at mid-day, during normal muscular activity, has a much smaller effect. For this reason a normal individual may pass a urine shortly after breakfast which might cause him to be rejected as a diabetic when examined for life insurance. Such cases would probably be passed as normal if a sample of the mixed 24 hours' specimen were examined. The effect of taking glucose varies with the dose and also with the time of administration. Apparently

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\* Cole, *Lancet*, 1913, ii., p. 859.

† Folin, *Journ. Biol. Chem.*, xxii., p. 327.

‡ Benedict and Osterberg, *Journ. Biol. Chem.*, xxxiv., p. 195.



glucose is tolerated better on an empty stomach than when taken with an ordinary meal. In general, it might be stated that a normal individual should be able to absorb 50 grams. of glucose on an empty stomach without showing any increase in the amount of sugar excreted in a given time. An important result of these researches is that the concentration of sugar in the urine is of much less significance than the amount passed per hour. Since urine always contains glucose, they suggest that the term "glycuresis" should replace "glycosuria," to indicate conditions characterised by an increased excretion of glucose in the urine.

There are two types of glycuresis, alimentary and persistent. Alimentary glycuresis is the condition in which the amount of sugar absorbed exceeds the amount that the individual is capable of assimilating. The limit varies with the individual, and is affected by a variety of pathological conditions. Persistent glycuresis is the condition when large amounts of sugar are excreted for a considerable length of time, and may be quite independent of the administration of carbohydrate food. The condition is known as diabetes mellitus. The urine is generally much increased in amount, of a high specific gravity, and pale in colour.

The classical test for sugar in urine is Fehling's (Ex. 97). It is not a reliable test. Not only is Fehling's solution reduced by certain constituents of normal urine, such as urates and creatinine; but also certain of these bodies, notably creatinine, form soluble compounds with cuprous oxide, and thus markedly interfere with the delicacy of the test. Also the urea of the urine is decomposed to ammonia, which dissolves cuprous oxide (see p. 106, note 5). Further, glucose is destroyed by boiling caustic soda, so that the presence of a small amount of sugar may escape detection.

Benedict's test (Ex. 100) is a great improvement. Owing to the substitution of sodium carbonate for sodium hydroxide the solution is not reduced by urates or creatinine. It does not give a positive reaction with the concentration of glucose normally present in urine, but is very sensitive for small increases beyond this. The author considers it the most reliable for general use; but owing to the fact that it is much more delicate than Fehling's, the result of a faintly positive test is not necessarily an alarming indication of abnormality.

Cole's test (Ex. 381) is more sensitive than Benedict's, and the manipulation has been so arranged as to ensure that it does not give a positive result with normal urines. It is of considerable value in detecting small variations

from the normal, but such cases should be examined by the application of Benedict and Osterberg's quantitative method. (Ex. 407.)

The osazone test serves to confirm the presence of glucose in doubtful cases, and especially to distinguish between glucose on the one hand and lactose and pentoses on the other.

The fermentation test is helpful in connexion with the recognition of lactose and glycuronic acid.

**380. Benedict's test.** To 5 cc. of Benedict's reagent (see p. 107) in a test-tube add eight drops of the urine. Boil vigorously for two minutes and allow to cool spontaneously. If glucose is present the entire body of the solution will be filled with a precipitate which may be red, yellow or green in colour, depending on the amount of sugar.

NOTE.—It is essential to add a small volume of urine. If too much be added the results are apt to be ambiguous. Even with the eight drops recommended, a slight precipitate of earthy phosphates may appear and simulate a feeble reduction.

**381. Cole's test for small amounts of glucose in urine.** In a dry boiling tube or large test-tube place about 1 gram. of adsorbent charcoal. Add 10 cc. of the urine, shake, heat to boiling and then cool under the tap. Shake at intervals for 5 minutes. Filter through a small paper into a dry test-tube. To the filtrate add 4 drops of pure glycerol and 0.5 gram. of anhydrous sodium carbonate. Shake and heat to boiling. Maintain the boiling for exactly 50 secs. Immediately add 4 drops of a 5 per cent. solution of crystalline copper sulphate, shake to mix and allow the tube to stand without further heating for one minute. With normal urine the fluid remains blue. If glucose is present to the extent of 0.03 per cent. above the normal amount in urine the blue colour is discharged and a yellowish precipitate of cuprous hydroxide forms.

NOTES.—1. Treatment with adsorbent charcoal removes practically the whole of the urates, creatinine and pigments that interfere with Fehling's test. It also adsorbs so much of the normal amount of glucose present that the filtrate from normal urine fails to give a reduction.

2. 0.5 gram. of anhydrous sodium carbonate is carried by about  $\frac{1}{4}$  the length of a large blade well piled up once.

3. Should the specific gravity of the urine exceed 1025 it is advisable to use 5 cc. of the urine + 5 cc. of water.

4. The test is not given by chloroform nor by glycuronates: it is given by pentoses.

5. Should there be any reason to suspect lactose the procedure should be modified as follows: treat 20 cc. of the urine with 1 gram. of charcoal as described above. Treat the whole of the filtrate with another gram. of charcoal and repeat the process. To 5 cc. of this filtrate add the glycerol and sodium carbonate and proceed as above directed. A reduction indicates the presence of glucose, the whole of any lactose up to even 1 per cent. being removed by this double adsorption, whilst 0.04 per cent. of glucose in the original urine still shows in the filtrate.

**382. Fehling's test.** Boil 3 to 5 cc. of Fehling's solution (see p. 106) to ascertain whether the Rochelle salt has been decomposed into reducing substances. If no reduction occurs boil the same volume of urine in another tube. Reboil the Fehling's solution and mix the two. Allow the tube to stand without further heating. If any appreciable amount of glucose is present a red or yellow precipitate will appear.

NOTE.—Prolonged boiling of the urine with Fehling's solution is very apt to lead to the formation of a greenish-yellow precipitate owing to the action of the strong alkali on the normal urinary constituents.

**383. Phenylhydrazine test.** Treat 10 cc. of urine with 1 cc. of strong acetic acid. Add enough phenylhydrazine hydrochloride to cover a sixpenny piece and twice this bulk of solid sodium acetate. Dissolve by the aid of heat and filter. Place the filtrate in a tube and immerse this in a boiling water-bath for 30 to 60 minutes. Turn out the flame and allow the tube to cool without removing it from the bath. Examine the deposit microscopically for the characteristic crystals of glucosazone (see p. 110).

NOTE.—With small amounts of glucose the crystals are apt to separate in small spherical clusters.

**384. Fermentation test.** Fill a test-tube with urine and then transfer the fluid to a mortar. Add a piece of washed yeast about the size of a bean and pound it up with the urine. Transfer the mixture to the test-tube and invert, placing the open end under mercury or urine contained in a small dish. Clamp the tube in position, and allow it to stand for at least eighteen hours in a warm place. If glucose is present in the urine there is an accumulation of gas ( $\text{CO}_2$ ) at the top of the tube.

NOTES.—1. Lactose, pentoses and glycuronic acids are not fermented by pure yeast.

2. A special apparatus called Einhorn's saccharometer has been devised to enable the test to be applied conveniently. Also the volume of  $\text{CO}_2$  formed, and the percentage of glucose present can be roughly determined by means of it.

### 7. *Fructose (laevulose).*

Fructose occasionally occurs in the urine, sometimes being accompanied by glucose. The significance of fructosuria is not yet clear.

385. **Seliwanoff's test** (Borchardt's modification). To a few cc. of urine in a test-tube add an equal volume of 25 per cent. hydrochloric acid and a speck or two of resorcin. Heat to boiling, cool under the tap, and transfer to an evaporating dish. Make the reaction alkaline by means of solid sodium hydroxide and return it to a test-tube. Add 3 cc. of acetic ether (ethyl acetate) and shake. A yellow colouration in the acetic ether indicates the presence of fructose.

### 8. *Pentoses.*

Pentoses, that is carbohydrates with 5 carbon atoms, appear in the urine in three conditions, alimentary, persistent or true pentosuria, and admixed with glucose in cases of glycuresis.

Alimentary pentosuria is sometimes seen after the ingestion of considerable quantities of certain fruits, as prunes, cherries, grapes and plums. The sugar found varies, but is usually *d*-arabinose. In true pentosuria it is *dl*-arabinose. Its origin and significance have not yet been clearly established.

Pentoses are indicated when the urine gives a positive Benedict's or Cole's test, but a negative fermentation test. The results of the phenyl-hydrazine test are variable, the osazones being somewhat soluble. The two colour reactions described are also given by glycuronic acid, which can, however, be demonstrated by Ex. 392.

386. **Tollen's test.** To 5 cc. of urine add an equal volume of strong hydrochloric acid and a little phloroglucin (a piece about the size of a pea) and heat the mixture on a boiling water bath. A cherry-red colour develops and the solution shows an absorption band between D and E. On cooling a dark precipitate separates

out. On dissolving this in strong alcohol, the alcoholic solution shows the colour and absorption band of the original mixture.

387. **Bial's orcin test.** To 2 — 3 cc. of urine add 4 — 5 cc. of Bial's reagent and heat till boiling commences. A green colour or the formation of a green precipitate indicates pentoses. The solution shows two absorption bands, one in the red between B and C and the other near the D line.

NOTE.—Bial's reagent consists of 1 to 1.5 gram. orcin, 500 cc. of concentrated hydrochloric acid, and 30 drops of a 1 per cent. solution of ferric chloride.

### 9. *Lactose.*

Lactose is found in the urine of women during pregnancy, during the nursing period, and soon after weaning. The amount in the urine varies, but rarely exceeds 1 per cent. The excretion usually reaches its maximum 2 to 4 days after parturition.

The only satisfactory method of demonstrating the presence of lactose in urine is by the author's test. The mucic acid test is inconvenient and not very reliable.

388. **Cole's test for lactose.** To 1 gram. of good charcoal add 25 cc. of the suspected urine, mix by shaking, boil for a few seconds, cool thoroughly, and shake at intervals for 10 minutes. Filter through a small paper or use a filter pump. When the charcoal has completely drained transfer it to a porcelain dish containing 10 cc. of water and 1 cc. of glacial acetic acid. This is best done by opening the paper, holding it by the clean half and moving it about in the liquid. The greater part of the charcoal is thus removed from the paper. Stir the charcoal with a glass rod and transfer the mixture to a boiling tube. Heat to boiling for about 10 seconds and filter the hot solution through a small paper into a test-tube containing as much solid phenyl-hydrazine-hydrochloride as will lie on a shilling, and twice this amount of solid sodium acetate. Mix thoroughly and filter from any insoluble oily residue. Place the tube in a boiling water bath and leave it there for 45 minutes. Remove the tube and allow it to stand at room temperature for at least one hour. It is advisable to allow it to stand longer if possible.

Lactosazone crystallises in characteristic clumps with projecting spines ("hedge-hog" crystals). It can be recrystallised by filtering through a small paper, washing with a small amount of distilled water, and then passing about 4 cc. of boiling water through the paper into a clean tube. The filtrate is boiled and passed through the paper two or three times, boiling between every filtration. On allowing the solution to stand, typical crystals of the osazone separate out.

NOTE.—The only charcoal that is of the slightest use for this test is the pre-war "Blood charcoal" of Merck.

389. **Mucic acid test.** 100 cc. of the urine and 20 cc. of pure concentrated nitric acid are evaporated in a wide and rather shallow beaker on a boiling water bath in a fume chamber. The evaporation is continued until the fluid becomes clear, and brown fumes are no longer evolved. The total volume is then about 20 cc. Remove the beaker from the bath and transfer the contents to a smaller beaker, washing out with a small amount of distilled water. Allow to stand over-night in a cool place. The formation of a white crystalline mass of mucic acid indicates the presence of lactose in the urine. Dilute the fluid, collect the crystals on a small filter and wash with cold water. Microscopically the crystals are seen to be very pointed prisms with oblique angles. The melting point is  $213^{\circ} - 215^{\circ}$  C. It can be weighed and titrated with standard alkalis, its equivalent weight being 105.

NOTE.—Mucic acid is  $\text{COOH}(\text{CHOH})_4\text{COOH}$ .

#### 10. *The Acetone bodies.*

The acetone bodies found in urine in certain forms of the condition known as "acidosis" are

Acetone.  $\text{CH}_3\text{CO.CH}_3$ .

Aceto-acetic acid.  $\text{CH}_3\text{CO.CH}_2\text{COOH}$ .

$\beta$ -oxy-butyric acid  $\text{CH}_3\text{CH}(\text{OH}).\text{CH}_2\text{COOH}$ .

The acetone must be regarded as a decomposition product of aceto-acetic acid, which loses  $\text{CO}_2$  on being heated. The excretion of the acetone bodies depends on the inability of the tissues to oxidise completely the

fatty acids generally derived from the fats, but sometimes from certain of the amino-acids formed in the metabolism of proteins. The condition that usually gives rise to acetonuria is the inability of the tissues to obtain or to utilise an adequate amount of glucose. Thus these acetone bodies are excreted in starvation, on a diet of fats with a limited amount of protein, in certain fevers, severe anaemias and after phosphorus poisoning, and finally in diabetes mellitus, in which condition the tissues are unable to utilise the glucose provided.

It is a remarkable fact that the urine passed just after an operation with a volatile anaesthetic nearly always contains a considerable amount of the acetone bodies. The author has confirmed Piper's statement that this post-operative acidosis is much decreased by the previous administration of dried pancreatic tissue ("pancreatin"). It is also noteworthy that the acidosis is partially dependent on the previous dietetic treatment of the patient, a fair dose of a carbohydrate such as lactose having a very beneficial effect. In some cases, especially after the use of chloroform, the acidosis may recur. This condition of "delayed chloroform poisoning" is very apt to lead to fatal results.

In children recurrent vomiting may be associated with acidosis. In many of these cases the condition of the patient is similar to that seen in peritonitis or appendicitis. Examination often reveals a deep pain on pressure over the pancreas. Operative interference in such cases without treatment of the acidosis is an extremely risky undertaking. The recognition of the condition of acetonuria is therefore of great importance.

The simplest and most reliable test for acetone and aceto-acetic acid is Rothera's. So far a simple test for  $\beta$ -oxy-butyric acid has not been introduced. But as this is only found together with aceto-acetic acid, its presence is only of qualitative significance.

The methods of estimation of the acetone bodies are described on pages 349 to 354.

389A. **Rothera's test for acetone and aceto-acetic acid.** To 10 cc. of the urine add an excess of solid ammonium sulphate, so that the urine is completely saturated. Then add two or three drops of a freshly prepared 5 per cent. solution of sodium nitroprusside and 2 or 3 cc. of concentrated ammonia. Mix and allow to stand undisturbed for at least thirty minutes. A characteristic permanganate colouration, that may only develop above the layer of undissolved crystals, indicates the presence of acetone or aceto-acetic acid.

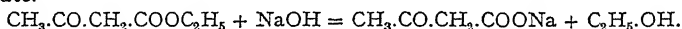
NOTES.—1. The test is much more sensitive for aceto-acetic acid than for acetone. The reaction with acetone can just be detected in a dilution of 1 in 20,000, whilst aceto-acetic acid shows it in a 1 in 400,000 dilution.

2. The intensity of the colour and the rate at which it develops vary with the concentration. Kennaway\* suggests the following scale:—(1) quick-strong; (2) slow-strong; (3) quick-weak; (4) slow-weak. A "quick-strong" reaction shows a deep permanganate colour within a few seconds, and indicates the presence of at least 0.25 per cent. aceto-acetic acid. A "slow-weak" reaction may show no pink for some minutes, and the amount present is probably less than 0.0005 per cent.

3. Though aceto-acetic acid gives a vivid reaction, aceto-acetic ester actually inhibits the reaction when the free acid or acetone are present.

4. For the sake of experience it is advisable to practise the tests on urines containing varying amounts of acetone and aceto-acetic acid. The latter is prepared from the ester by the following method of Hurtley.

Into a beaker weigh out 13 grams. (1.10 molecule) of aceto-acetic ester. Add 100 cc. of N. soda (1.10 molecule) diluted with about 300 cc. of distilled water. Transfer to a 500 cc. measuring flask and make the volume up to 500 cc. with distilled water. Allow the solution to stand for at least 24 hours at room temperature. The ester is completely hydrolysed to sodium aceto-acetate.



The solution obtained corresponds to a 2 per cent. solution of aceto-acetic acid.

390. **Gerhardt's test for aceto-acetic acid.** A. To 5 cc. of the urine in a test-tube add ferric chloride solution, drop by drop, till no further precipitate of ferric phosphate is formed. Filter. To the filtrate add some more ferric chloride. A Bordeaux-red colour indicates aceto-acetic acid.

NOTE.—A similar colour is given by a large number of substances, such as salicylic acid, and the bodies excreted after the administration of aspirin, antipyrin, thallin, etc. The majority of these substances are not destroyed by boiling, whereas aceto-acetic acid is converted into acetone. The reaction is not obvious when less than 0.07 per cent. of aceto-acetic acid is present.

\* *Guy's Hospital Reports*, lxvii., p. 161.



B. If A is positive shake 50 cc. of urine and 3 drops of strong sulphuric acid with ether. Pipette off the ether and treat it with very dilute ferric chloride. The lower layer becomes coloured violet. Add more ferric chloride. The colour changes to a Bordeaux-red.

NOTE.—It is advisable to shake the acidified urine first with chloroform or benzene, to extract salicylic acid.

391. **Hurtley's test for aceto-acetic acid.** To 10 cc. of urine add 2.5 cc. of concentrated hydrochloric acid and 1 cc. of a freshly prepared 1 per cent. solution of sodium nitrite. Shake and allow to stand for two minutes. Now add 15 cc. of strong ammonia, then 5 cc. of a 10 per cent. solution of ferrous sulphate. Shake, pour into a large boiling tube and allow to stand undisturbed. A violet or purple colour slowly develops if aceto-acetic acid be present. The speed at which the colour develops depends on the concentration of aceto-acetic acid. With small amounts the colour may not develop for about 5 hours. The test shows in a dilution of 1 in 50,000.

## II. *Glycuronic Acid.*

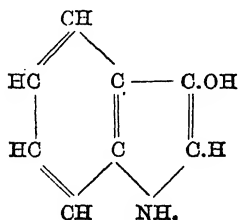
Glycuronic acid,  $\text{CHO}(\text{CHOH})_4\text{COOH}$ , is not found free in the urine. It is found conjugated with certain drugs, or with substances formed from these in the body. These conjugated glycuronates are excreted after administration of chloral, camphor, naphthol, menthol, phenol, morphine, oil of turpentine, antipyrin, etc. The free and conjugated acids are reducing substances, but are not fermentable. They give the reactions for the pentoses, but can be distinguished by the test given below.

392. **Tollen's test for glycuronates.** To 5 cc. of the urine in a rather wide test-tube add .5 to 1 cc. of a 1 per cent. solution of naphthoresorcin in alcohol and 5 to 6 cc. of strong hydrochloric acid. Heat slowly to boiling point and keep boiling for 1 minute, shaking the tube the whole time. Set the tube aside for 4 minutes, then cool under the tap and shake with an equal volume of ether. The ether is coloured violet to red, and when examined spectroscopically shows two bands, one on the D line, and one to the right of it.

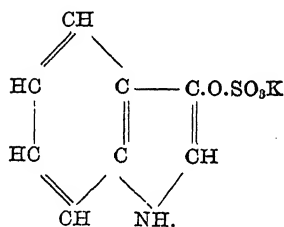
12. *Indican.*

Indican is the potassium salt of indoxyl sulphuric acid, and is thus one of the ethereal sulphates (see p. 281).

Indoxyl is



Indican is



Indoxyl arises from the bacterial decomposition of tryptophane in the intestine, thus differing from the other ethereal sulphates which are normal tissue metabolites (see p. 282). The excretion of indican is of importance as a measure of the amount of putrefaction occurring, generally in the intestine, but sometimes in a large abscess.

393. **Jaffe's test.** Treat 5 cc. of urine with a rather larger volume of concentrated hydrochloric acid and about 2 cc. of chloroform. Add a single drop of 5 per cent. potassium chlorate and mix. Allow the chloroform to settle and examine its colour. If it be blue, indican is present. If not, add another drop of the chlorate and mix again. If no blue colour be found in the chloroform, indican is absent.

NOTE.—The extraction with chloroform is best done by repeatedly pouring the mixture from one tube to another. It is essential to add at least an equal volume of strong hydrochloric acid to liberate free indoxyl. This is oxidised to indigo blue, which is soluble in chloroform.

Horse's and cow's urine nearly always contain indican. If procurable they should be used for the sake of experience.

### L. Urinary Sediments.

For the proper examination of these substances a hand centrifuge is desirable. The sediment obtained should be examined microscopically, and chemically if necessary.

The sediments obtained are either organised or unorganised. Organised sediments consist of casts of the renal tubules, epithelial cells from different parts of the urinary tract, pus, blood cells, spermatozoa, parasites, etc. It is not thought advantageous to describe them in this book.

Unorganised sediments vary with the reaction of the urine. The more common varieties are given below.

#### *In acid urine.*

**Uric acid :** light yellow to dark reddish-brown in colour. Crystalline form very varied : rhombic prisms, wedges, rosettes, dumb-bells, whetstones, butchers' trays, etc. Soluble in sodium hydroxide and reprecipitated by hydrochloric acid.

**Urates :** pinkish, soluble on warming, sometimes amorphous, sometimes crystalline, as "thorn-apples," fan-shaped clusters of prismatic needles.

**Calcium oxalate :** octahedra, with an envelope-like appearance (squares crossed by two diagonals); also in dumb-bells. Insoluble in acetic acid, easily soluble in hydrochloric acid.

**Calcium hydrogen phosphates** (stellar phosphates): in rosettes of prisms and in dumb-bells. Rather rare.

**Cystine :** colourless hexagonal plates, soluble in ammonia, insoluble in acetic acid. Very rare.

*In alkaline urine.*

**Ammonium magnesium phosphate** (triple phosphate): colourless prisms ("coffin-lids" and "knife-rests") or feathery stars. Easily soluble in acetic acid.

**Alkaline earthy phosphates** of calcium and magnesium: amorphous. Insoluble on warming and in alkalies, soluble in acetic acid.

**Calcium hydrogen phosphate**: see above.

**Calcium carbonate**: dumb-bells or spheres with radiating structure.

**Ammonium urate**: yellow, or brownish amorphous masses, or shewing "thorn-apple" crystals. Soluble on warming.

## CHAPTER XIII.

### THE QUANTITATIVE ANALYSIS OF URINE.

To determine the nature of the metabolic processes in the body a sample of the measured 24 hours' urine must be analysed. In taking the 24 hours' urine it is best to finish with that voided after the night's rest. The total collected during the 24 hours is mixed and carefully measured. The analyses should be performed as soon as possible, owing to the risk of bacterial decomposition of certain of the constituents. Should it be necessary to postpone the analyses an antiseptic should be added. Toluol is the best to use. Chloroform must not be used in any case, since it is decomposed by alkalis and has a marked effect on certain processes.

The analyses performed will vary with the nature of the case that is being investigated, and the time and apparatus at the disposal of the analyst. It is of the utmost importance for the student to acquire skill in the conduction of a complete analysis, and it is to be hoped that the specially selected methods described below will be practised until satisfactory results can be obtained in the minimum of time. By careful organisation in a well-equipped laboratory it is possible to estimate total nitrogen, urea, ammonia, acidity, uric acid and creatinine in three hours.

The remarks on pages 380 to 382 on the use of pipettes, etc., should be carefully studied before undertaking any analytical work. For the method of returning the results of analyses see the form on page 378.

#### A. Total Nitrogen by Kjeldahl's method.

*Principle.* The nitrogenous compounds in a given volume of urine are converted into ammonium sulphate by boiling with sulphuric acid, copper sulphate being added to aid the oxidation, and potassium sulphate to raise

the boiling point. The mixture is diluted with water, made alkaline by the addition of sodium hydroxide and the ammonia distilled into a measured amount of standard acid. The amount of this neutralised by the ammonia is found by subsequent titration with standard alkali. Knowing the amount of ammonia formed from the volume of urine taken, the percentage of nitrogen can be readily calculated.

The technique adopted varies with the material and with the inclinations of the operator. There are four main methods of distillation, viz., by direct boiling, by steam, by boiling with alcohol, and by the author's method of combined boiling with alcohol and aeration.

The main objection to direct boiling is that the mixture is apt to bump very violently, and that a certain loss of ammonia may occur when adding the alkali. The bumping is much less with potash than with soda, but the price is prohibitive.

Steam distillation is much safer, and, although it is slow, it needs very little attention. It is the best method when considerable amounts of sulphuric acid have to be used for the incineration.

Distillation with alcohol is very smooth and relatively rapid, for the alcohol boils quickly and carries the ammonia over with it.

The combination of aeration with alcohol distillation (Ex. 397) was originally devised for the estimation of very small amounts of nitrogen, but it is so rapid and accurate that the author now uses it for the estimation of total nitrogen in urine. Either 0.5 or 1 cc. of the urine is measured with an Ostwald pipette, the incineration is completed in 15 mins., and the result is obtained in less than 30 mins. The results agree exactly with those obtained when 5 cc. of urine are taken.

*Standard acids and alkalis and indicators.* It is essential to use  $\text{CO}_2$ -free soda for the back titrations. The end point is extremely sharp if methyl red

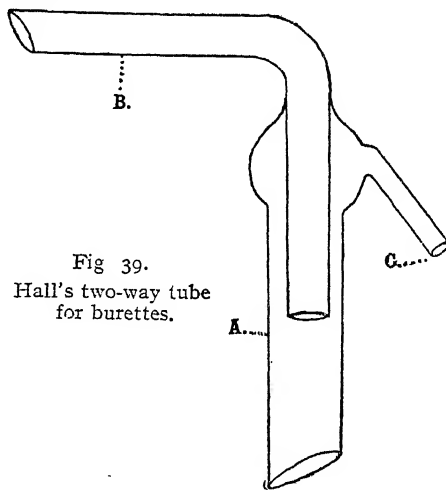


Fig. 39.  
Hall's two-way tube  
for burettes.

is used as an indicator. With ordinary soda the yellow tint obtained when the solution is just alkaline soon changes to a pink, and there is great uncertainty as to how far to proceed with the titration. This is fatal to good results. With  $\text{CO}_2$ -free soda, on the other hand, the change of tint is brought about by the addition of less than a drop of 0.02 N. soda, and is permanent for a considerable time. The titration can be conducted by artificial light, which is not the case with methyl orange. The preparation of  $\text{CO}_2$ -free soda is described on page 26. The strength required varies with the method adopted. For ordinary work the author prefers to use about 0.1 N. strength, but for the micro-

method it is better to use 0.03 to 0.05 N. As a rough guide it may be stated that the strong soda prepared according to the directions on page 25

is about 10 N. A paraffined bottle, a grease-free burette (see p. 385) and a good supply of recently boiled and cooled distilled water being ready, the bottle is nearly filled with the water, the volume of which is noted. The requisite amount of the strong  $\text{CO}_2$ -free soda is added, the solution well mixed, and the burette and soda-lime tubes immediately fitted. It is advisable to seal all the joints with paraffin wax, to prevent the absorption of atmospheric  $\text{CO}_2$ , and to ensure the due filling of the burette by suction. It is difficult to fit the upper stopper to an ordinary burette. To overcome this, Messrs. Baird and Tatlock are making burettes fitted with a wide top, similar to that shewn on page 130. Mr. H. W. Hall, of the Cambridge Biochemical Laboratory, has made it possible to use an ordinary burette by the construction of the two-way piece shewn in fig. 39. This can be obtained direct from Mr. Hall or from Messrs. Baird and Tatlock.

The exact concentration of the soda is determined by titration of a weighed amount of acid potassium phthalate, or by the use of an acid of known normality.

The acid employed can be hydrochloric or sulphuric. In the case of the aeration methods it is advisable to use the latter, to avoid any risk of loss by volatility. The strength should be somewhat greater than that of the alkali.

#### Method of titration.

A great deal of time is saved if the acid be measured from a burette rather than with a pipette. The initial reading is noted, the approximate amount required is run in, and the burette allowed to stand until the final titration is performed. The soda is then run in until the solution goes yellow, a little acid is added until a pink tint appears. The sides of the vessel are washed down with a little distilled water, and the titration completed by the addition of a few drops of the soda. In this way, being relieved from the anxiety that one may overshoot the mark with the alkali, it is not necessary to run the whole of the alkali in drop by drop, which may take a considerable time. The only danger is that of allowing insufficient time for proper drainage of the alkali burette.

#### Calculation.

1 gram.-molecule of acid neutralises 1 gram.-molecule of ammonia.

So 1,000 cc. of N.HCl (or other acid) neutralises 17 grams.  $\text{NH}_3$  and are equivalent to 14 grams. of Nitrogen.

So 1 cc. of N. acid  $\equiv$  14 mgms. Nitrogen.

So 1 cc. of (A)  $\times$  N. acid  $\equiv$   $14 \times (A)$  mgms. Nitrogen ("acid equivalent.")

Suppose that the soda employed be (S) normal,

Then 1 cc. soda  $\equiv \frac{(S)}{(A)}$  cc. acid ("alkali-acid ratio").

Let the amount of acid used be (a) cc., and the amount of soda be (s) cc.

Then (s) cc. alkali =  $\frac{(S) \times (s)}{(A)}$  cc. acid.

So volume of acid neutralised by the ammonia distilled over is

$(a) - \frac{(S) \times (s)}{(A)}$  cc., and the amount of nitrogen is

$$\left[ (a) - \frac{(S) \times (s)}{(A)} \right] \times 14 \times (A) \text{ mg.}$$

A good deal of time is saved if the acid and alkali be labelled with the logarithms of the "acid-equivalent" (acid log.) and of the "alkali-acid ratio" (alkali log.) respectively.

The following example should be carefully studied.

Acid employed was 0.0476 N. sulphuric.

So 1 cc.  $\equiv$  0.0476  $\times$  14 mgms. nitrogen.

$$\log. 0.0476 = 2.6776$$

$$\text{add log. } 14 = 1.1461$$

$$\text{So "acid-log" } = 1.8237$$

Alkali employed was 0.0421 N. soda.

$$\text{So 1 cc. soda } \equiv \frac{0.0421}{0.0476} \text{ cc. acid.}$$

$$\text{Log. } 0.0421 = 2.6243$$

$$\text{Subtract log. } 0.0476 = 2.6776$$

$$\text{So "alkali-log." is } 1.9467$$

0.5 cc. of urine taken.

Amount of acid taken was 20.12 cc., and this required 13.6 cc. of alkali for back titration.

$$\text{Log. of } 13.6 = 1.1335$$

$$\text{Add the alkali log } 1.9467$$

$$\text{Anti-log. of } 1.0802 \text{ is } 12.03.$$

So 20.12 - 12.03 = 8.09 cc. of acid have been neutralised by the ammonia formed from 0.5 cc. of urine.

$$\text{Log. of } 8.09 \text{ is } .9079$$

$$\text{Add the acid log. } 1.8237$$

$$\text{Anti-log. of } .7316 \text{ is } 5.39.$$

So 0.5 cc. of urine contain 5.39 mg. total Nitrogen.

So 100 cc. of urine contain 1.078 gram. total Nitrogen.

No allowance has been made for the **blank determination**, but this should not be neglected, especially when using the micro-method. The blank determination is made with all the materials used for an ordinary analysis, distilled water being taken instead of urine. Unless the reagents are of very poor quality, the amount of nitrogen found should be very small. This must be deducted from the amount found in the volume of urine taken. An example is given on page 263.

394. **Kjeldahl's method (distillation by boiling).** Into a clean, dry, round-bottomed flask of "Duro" glass A (500 cc. capacity, with a narrow neck 8 inches in length) place 5 to 10 grams. potassium sulphate, 0.5 cc. of saturated copper sulphate solution, 5 cc. of urine (accurately measured) and 10 cc. of concentrated sulphuric acid, free from nitrogen. Place the flask in the fume-chamber (or use the fume-absorber, described on page 387), and heat by means of a low flame for 10-15 minutes, then boil briskly for 45 minutes or longer. The solution must be heated for at least 15 minutes after it has lost every trace of dark colour. Any particles of carbonaceous matter that adhere to the sides of the flask must be



washed down into the acid by carefully shaking the flask. When cool add 250 cc. of ammonia-free distilled water, 3 or 4 pieces of broken porous pot, and cool under the tap. Into an Erlenmeyer flask, E, of about 400 cc. capacity, place 20 cc. of standard sulphuric acid, about 0.2 N.

This flask is then placed on an adjustable stand, so arranged that the lower end of the tube D dips below the surface of the acid in E. The bulb in D is to decrease the risk of the acid in E being sucked back by a sudden cooling of A during the distillation. D is connected to a condenser C. The best pattern is Davies', which is shewn in fig. 40.

To the flask A add 35 to 40 cc. of 40 per cent. sodium hydroxide, pouring it down the neck and wall of the flask so as to form a bottom layer; loss of ammonia is thus prevented.

Fit the glass tube B into the neck of A by means of a well-fitting rubber stopper. The special bulb on B is to prevent any of the alkaline fluid bumping over into the distillate.

Mix the contents of A by shaking and immediately connect up B with C by means of another well-fitting rubber stopper. Heat the mixture in A to boiling by means of a free flame from a Bunsen burner provided with a rose-top. Allow the fluid to boil till at least half the total volume of fluid has distilled over, lowering E from time to time, so that D does not dip too far under the acid. Finally, lower E so that the tube no longer dips under the surface and continue the boiling for another minute or two to wash down any of the standard acid

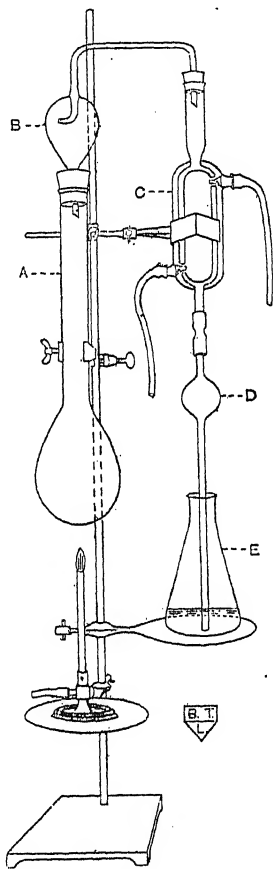


Fig. 40. Kjeldahl apparatus. Distillation by direct boiling.

that may have been sucked up into the tube or bulb. Wash down the exterior of the lower end of D with a jet of distilled water, allowing the washings to run into E.

To the fluid thus obtained add a few drops of a 0.02 per cent. solution of methyl red and titrate with standard  $\text{CO}_2$ -free sodium hydroxide, which may be between 0.1 and 0.15 N.

*Calculation*, see pages 323 and 324.

395. **Kjeldahl's method (steam distillation).** The incineration is conducted as described in the previous exercise, but a smaller Kjeldahl flask may be used if desired. After the fluid

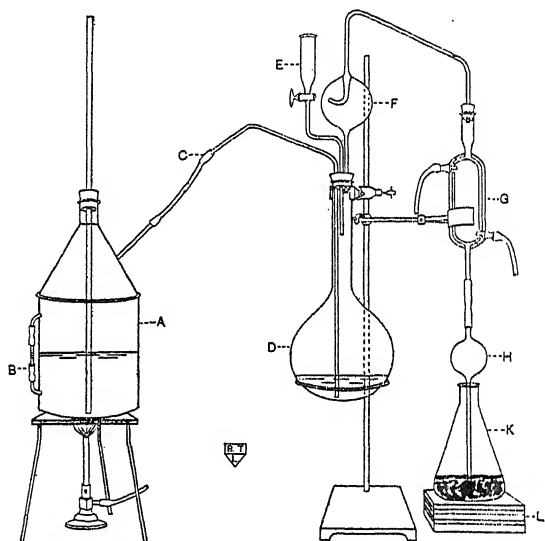


Fig. 41. Kjeldahl apparatus. Steam distillation.

has cooled, add 50 cc. of distilled water, shake round well and transfer the solution to D, a round-bottom flask of 1 or 2 litres, with a neck sufficiently wide to carry a well-fitting rubber stopper that will allow 3 tubes to pass through, as shewn in the figure. Wash out the Kjeldahl flask twice more, using about 25 cc. of distilled water each time. Measure the requisite amount of standard acid into K, and assemble the apparatus. Arrange the wooden blocks, L, so that the end of the delivery tube, H, just dips under

the surface of the acid. ~~(It is incorrectly drawn in the figure.)~~ E is a tap-funnel containing 40 per cent. soda. G is a condenser, the double-surface variety being the most efficient.

The water in the copper vessel A has previously been vigorously boiled for at least ten minutes to ensure the removal of any ammonia. Remove the flame for a moment and connect the exit pipe of the boiler to C by rubber tubing. Replace the burner. Run in the strong soda from E until the solution is definitely alkaline, as can be seen by the fluid turning blue, due to the formation of cupric hydroxide. The distillation must be allowed to proceed for at least 45 minutes. It is safer to allow an extra half-hour. The only attention necessary is to see that there is a sufficient amount of water in the boiler and that the flask K is at the right height.

At the end of the operation, remove the blocks from under K, so that H does not dip into the acid. After a few minutes, remove the flame, wash down the interior and exterior of H into K, and titrate as described on p. 323.

*Calculation*, see p. 323.

396. **Kjeldahl's method (alcohol distillation).** Into a 500 cc. Kjeldahl flask of "Duro" glass measure 2 cc. of the urine, using an Ostwald pipette (fig. 51). Add 3 cc. of pure concentrated sulphuric acid, 2 grams. of potassium sulphate and 2 drops of saturated copper sulphate solution. Heat over a micro-burner, using a Folin's fume-absorber. The flame should be about half an inch in height, and should play directly on the bottom of the flask to ensure boiling. Any particles of carbonaceous matter that form on the side of the flask must be rinsed down into the acid. The heating must be continued for 5 to 10 minutes after the solution has turned blue. Remove the flame and allow the solution to cool until the flask is only pleasantly warm to the hand. Add 20 cc. of distilled water from a measuring cylinder. This should be added rapidly, and the mixture immediately shaken to prevent the formation of a cake of potassium hydrogen sulphate. Cool under the tap. Add three or four pieces of broken porous pot and 15 cc. of 95 per cent. alcohol. Assemble the apparatus, seeing that the clamps are correctly adjusted, so that the rubber stoppers fit into the flasks without undue strain. G is a piece of glass rod, with the lower end flattened

out and bent up as shewn. It is passed up through the rubber stopper, and the upper end can then be flattened out, if desired, for convenience of manipulation. The tube is drawn up until the flange is about  $\frac{1}{4}$  inch from the exit tube B. This minimises the risk of any alkali being carried over by spurting. This risk, however, has been

found to be so small that it is hardly worth the trouble of fitting. B is joined to the tube to the condenser by a short piece of rubber tubing. This allows the operator to shake A, and, by removing the strain, decreases the risk of breaking the glass parts. The standard acid is measured into E, a 250 cc. Erlenmeyer flask. 20 cc. of 0.1 N. acid is usually ample. The wooden blocks, F, should be so arranged that the lower end of D only just dips below the surface of the acid. Now remove A and run in 13 or 14 cc. of 40 per cent. soda, running this gently down the lower part of the neck and sides of the flask, so that the soda sinks to the bottom of the fluid and the risk of the loss of ammonia is minimised. The flask should be held in a nearly horizontal position during this operation; it should be raised to the vertical cautiously, so as to prevent mixing of the two layers. Re-assemble the apparatus, seeing that the two rubber

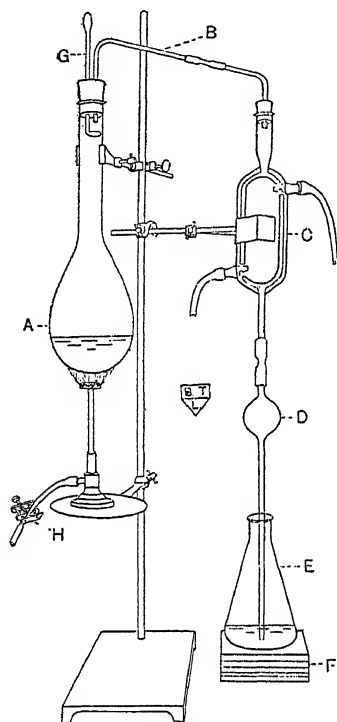


Fig. 42. Kjeldahl's method.  
Cole's apparatus for alcohol  
distillation.

stoppers are firmly held. Have ready a Bunsen burner, with a rose-top, and a screw clip, H, fitted to the rubber tubing. See that the adjustable stand for the burner is at such a height that the top of the rose is about half an inch under the bottom of the flask. Turn on the water supply to the condenser. Unclamp the flask A and mix its contents by shaking, taking care that the rubber stoppers

are not loosened. Place the burner in position and gently agitate the flask until the fluid commences to boil. The flask can then be clamped and the distillation allowed to continue for 15 minutes. Remove the wooden blocks F, and then remove the flame. Remove the rubber stopper from the upper end of the condenser and wash the latter down into E with a jet of distilled water. Wash down the exterior of D also, add a few drops of methyl red and titrate with the  $\text{CO}_2$ -free soda, as described on page 323. The soda can be between 0.05 and 0.1 N.

*Calculation*, see p. 323.

397. **Micro-Kjeldahl (Cole's method).** Into a 300 cc. Kjeldahl flask measure 0.5 or 1 cc. of the urine, using an Ostwald pipette (fig. 51). Add 2 cc. of pure concentrated sulphuric acid 2 drops of saturated copper sulphate, and 1 gram. of pure potassium sulphate. Clamp the flask over a micro-burner, having a flame about  $\frac{1}{2}$  inch in height, just touching the bottom of the tube, and insert a Folin's fume absorber (fig. 54) into the mouth of the flask. Continue the gentle boiling for at least 5 minutes after the solution has lost all trace of its dark colour and has turned light blue. Allow to cool, add 20 cc. of distilled water and 12 cc. of alcohol, and proceed exactly as described in the last paragraph of Ex. 311.

## B. The Estimation of Ammonia.

The ammonia of urine normally exists as ammonium salts of weak acids. In cases of cystitis, however, the urine is nearly always alkaline, owing to the conversion of some of the urea to ammonium carbonate by various micro-organisms. This change may occur after the urine has been passed owing to bacterial contamination from the air, etc. For this reason it is essential that a little toluol should be added to the vessel in which the urine of the 24 hours is being collected, that it should be kept in a cool place and that the estimations should be made as soon as possible.

A great many methods have been proposed for the estimation of ammonia in urine. Folin has introduced them at a rate which is almost alarming. Nearly all his later methods are colorimetric, a most excellent modification of Nessler's solution having been elaborated. But the author's experience with large classes is that the majority of workers prefer to use a titration method if possible. Mainly for that reason, the only three methods described here are Folin's original macro-method, Van Slyke's modification of it, and the formol method. The latter, however, gives the sum of ammonia and the amino-acids, and the results obtained by it are only of approximate value for the ammonia figure. It will be described in connexion with the estimation of amino-acids.

Of the two methods described below, the author now always employs Van Slyke's, which is much more rapid than Folin's. The author is convinced that failures to obtain correct results are either due to inattention to essential details or to the use of an imperfect suction pump. An apparatus that supplies air under a good pressure is a most valuable adjunct to a modern biochemical laboratory.

### 398. The estimation of ammonia by Folin's method.

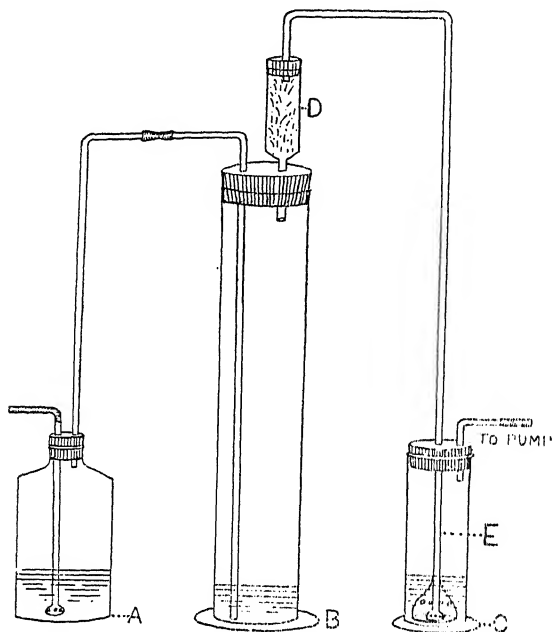


Fig. 43. Folin's apparatus for estimating ammonia.

- A. Wash bottle containing acid.
- B. Tall aerometer cylinder containing urine.
- C. Bottle containing standard acid to absorb ammonia from the air.
- D. Calcium chloride tube, loosely packed with cotton wool, to prevent any sodium carbonate being carried over into C.
- E. Folin's absorption tube, to bring the air into intimate contact with the acid.

Use the apparatus shewn above.\*

\* The parts of the apparatus can be obtained from Messrs. Baird and Tatlock (London).

Into C measure 20 cc. of standard sulphuric acid (about 0.1 N.) and a few drops of methyl red.

Into B measure 25 cc. of urine, add 5 or 6 drops of caprylic alcohol (to prevent foaming) and 2 grams. of anhydrous sodium carbonate. Connect up the apparatus at once, and draw air through for two hours.

Disconnect the apparatus, wash the tube E with distilled water into C, and titrate with  $\text{CO}_2$ -free sodium hydroxide (about 0.1 N.).

*Calculation.* Determine the percentage of nitrogen in the form of ammonia as described for Kjeldahl's method, p. 323. The result thus obtained is the mgms. of ammonia-nitrogen in 25 cc.

To convert this to grams. of ammonia per cent., multiply by

$$4 \times \frac{17}{14} \times \frac{1}{1000} = 0.00486 \text{ (log. } \bar{3}.6864\text{)}.$$

To find the ammonia in terms of cc. of 0.1 N. acid per cent., multiply the mgms. of ammonia-nitrogen in 25 cc. by  $4 \times \frac{10}{14} = 2.86 \text{ (log. } .456\text{c)}.$

### 399. Van Slyke's method.

*Principle.* 5 cc. of urine are made strongly alkaline with potassium carbonate, which decomposes the ammonium salts. The ammonia liberated is driven over by an air current into a measured amount of standard acid, which is subsequently titrated with standard alkali. The treatment of urine at room temperature with potassium carbonate does not lead to the formation of ammonia from urea, etc., as does boiling with caustic soda.

*Apparatus.* This is shewn in fig. 44.\* A is a wash bottle containing

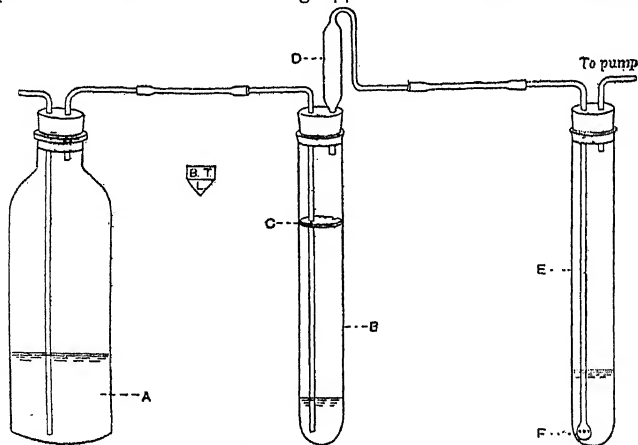


Fig. 44. Apparatus for estimation of ammonia and urea by Van Slyke's methods.

\* This can be obtained from Messrs. Baird and Tatlock (Ltd.), 14, Cross Street, Hatton Garden, London, E.C.

sulphuric acid (1 in 10) to remove ammonia from the air. B is a large thick-walled tube, 25 to 30 mm. by 200 mm. C is a sheet of rubber, about 2 mm. thick, cut from a rubber stopper. It fits loosely into B, and has a small groove cut at the side. It decreases the risk of an alkali foam being carried over into E, which is a tube similar to B. D can be made from a broken 5 cc. pipette and may be loosely filled with cotton or glass wool. F is a tube sealed at the lower end with holes bored in it, whilst still hot, with a hot needle. The tubes B and E are conveniently held by means of a heavy wooden block bored with two large holes. In place of the tube E, a 100 cc. flask with a wide neck may be substituted as shown in fig. 45. The advantage of the flask is that there is very little risk of the standard acid being carried over with the brisk air current necessary. The objection to it is that the depth of the acid layer being decreased there may be a danger of loss of ammonia. If the air current is a moderate one for the first two minutes this risk is very slight, and perfect results are obtained.

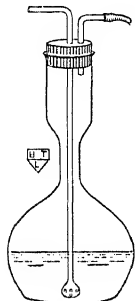


Fig. 45.

An efficient suction pump or blast pump is also required.

*Method.* (i.) Into B measure 5 cc. of the urine, and add 2 drops of caprylic alcohol to stop foaming. 4 or 5 drops of kerosene can be used, but it is not an efficient substitute.

(ii.) Into E measure 20 cc. of the standard sulphuric acid, which should be between 0.04 and 0.07 N, and then add a couple of drops of caprylic alcohol. If a flask is used it is advisable to add about 20 cc. of distilled water to give a deeper layer for absorption.

(iii.) Place 4 to 5 grams. of pure dry potassium carbonate into B, roughly measuring it with a suitable spoon. Immediately connect up the apparatus, taking care that D is joined to F, and not to the connexion for the pump. Turn on the water supply to the pump so that a rather slow air current is drawn through. After 2 to 3 minutes, turn on the water to full pressure and leave it for 12 more minutes.

(iv.) Gradually stop the pump and disconnect the tube E. Lift up the rubber stopper so that F does not dip into the acid, and wash down the interior of F with distilled water, using a fine jet. Repeat this twice, allowing time for proper drainage. Carefully wash down the exterior of F with distilled water, add 3 or 4 drops of methyl red and titrate with  $\text{CO}_2$ -free soda, which may be between 0.03 and 0.06 N., according to the directions given on page 323.

*Calculation.* Determine the percentage of nitrogen in the form of ammonia



as described for Kjeldahl's method, p. 323. The result thus obtained is the mgms. of ammonia-nitrogen in 5 cc. = A.

Mgms. of ammonia-nitrogen in 100 cc. = 20 A.

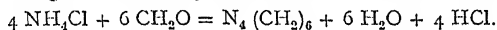
Grams. of ammonia-nitrogen in 100 cc. =  $A \times 0.02$ .

Grams. of ammonia in 100 cc. =  $A \times 0.02 \times \frac{17}{14} = A \times 0.0243$  (log. 2.3853).

cc. of 0.1 N. acid neutralised by  $\text{NH}_3$  of 100 cc. =  $20 A \times \frac{10}{14} = A \times 14.29$   
(log. 1.1549)

#### 400. C. The estimation of ammonia and amino-acids by formol titration (Cole's method).

*Principle.* Neutral ammonium salts react with an excess of neutral formaldehyde to give hexamethylene tetramine, the acid being liberated.



From the amount of alkali required to again make the solution neutral, the amount of ammonia can be estimated.

Neutral amino-acids also react with formol to give methylene amino-acids [see p. 69 (3)]. The result of the estimation therefore gives the sum of the ammonia and the amino-acids of the urine.

The method usually adopted is to neutralise the urine to phenol-phthalein, to add neutralised formol, which makes the fluid acid, and then to determine how much standard soda is again required to neutralise the mixture. The great difficulty encountered is that of determining the neutral point, and experience with large classes of students has revealed the fact that considerable variations in results are found, due to the indecision about the two end points.

As explained on p. 215, the author has overcome this difficulty by the use of the comparator shewn in fig. 27. The results obtained by untrained students now agree very closely.

*Method.* Use the comparator for large tubes described on p. 276.

Into tubes (2), (3) and (6) measure 20 cc. of the urine.

Into tube (1) measure 20 cc. of buffer solution  $P_H = 8.4$  (see p. 28).

Into tube (5) measure 20 cc. of buffer solution  $P_H = 8.5$ .\*

Into tube (4) place about 30 cc. of water.

To tubes (1), (3) and (5) add 10 to 20 drops of 0.5 per cent. phenol phthalein, adding exactly the same amount to each by the use of a dropping pipette (fig. 5). The amount necessary varies

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\* If only one buffer solution is used, as is done in the exercise on p. 215, it should be  $P_H = 8.45$ .

with the appearance of the urine, more being required for deeply pigmented urines.

Titrate with standard soda, which may be 0.1 to 0.2 N., as described in Ex. 322, until the colour as seen through Y is intermediate between that seen through X and Z. During the course of this titration, the standard soda is added to (2) and (6) and distilled water to (1) and (5), as described in Ex. 322. Usually a considerable precipitate of earthy phosphates appears in the three tubes that contain urine. The contents of these tubes must be well mixed by rotation or otherwise immediately before an observation is made.

Measure 5 cc. of commercial formaldehyde (40 per cent.) into a test-tube. Add one-third the number of drops of phenol phthalein added to the urine and then the standard soda, drop by drop, until a faint pink tinge is obtained. Add the whole of this solution to tube (3). Note that the pink tinge and the precipitate of earthy phosphates disappear, owing to the acidity developed. To tubes (2) and (6) add 5 cc. of water, to dilute the urinary pigment to the same degree as that in tube (3).

Read the burette containing the standard soda.

Titrate the contents of tube (3) with the soda, until the appearance at Y approaches that seen at X. To tubes (1), (2), (5) and (6) add the same volume of distilled water as the soda added in this last operation. Mix the contents carefully and complete the titration, so that the appearance at Y is intermediate between that seen at X and Z.

*Calculation.* If ( $a$ ) cc. of soda of normality ( $n$ ) are required to neutralise 20 cc. after the addition of the formol, then 20 cc. urine contain ( $a$ )  $\times$  ( $n$ )  $\times$  (14) mgms. of Nitrogen of ammonia and amino-acids. So 100 cc. urine contain ( $a$ )  $\times$  ( $n$ )  $\times$  (70) mgms. of (ammonia + amino-acid) Nitrogen. This amount, less 20 A (the mgms. of ammonia-Nitrogen determined in the previous exercise) is the mgms. of amino-acid Nitrogen in 100 cc. urine.

#### D. The Estimation of Urea.

The use of the enzyme urease (see p. 287) has rendered obsolete a large number of methods that had been devised for the estimation of urea in urine. The time required for D. Van Slyke's method is not much greater than that for the old hypobromite method, and the results obtained are accurate, whereas with hypobromite they are most unreliable.

The old hypobromite method has, however, been included again because of its convenience for the estimation of urea in McLean's "Urea Concentration

Test " of renal efficiency. Under the circumstances of the test, in which the urea concentration is determined in the urine collected during the second hour following the administration of 15 gms. of urea, the urea forms at least 90 per cent. of the total nitrogen of the urine. The hypobromite method in such cases is sufficiently accurate for clinical work, and since it demands very little equipment it is more convenient than the urease method for the majority of general practitioners.

#### 401. Van Slyke's method for urea.

*Principle.* A small volume of the urine is treated with Soya bean meal together with a certain amount of acid potassium phosphate to preserve the optimum reaction for the enzyme. The whole of the urea is rapidly converted to ammonium carbonate. An excess of potassium carbonate is added, and the ammonia formed from the urea, together with that from the preformed ammonium salts of the urine are driven over into standard acid and estimated in the way described in Ex. 399. The amount of ammonia being known, the percentage of urea can be found by difference.

*Apparatus.* This is exactly similar to that required for Ex. 399. A duplicate set should be obtained so that the ammonia and urea determinations can be conducted simultaneously.

*Soya bean meal.* It is cheaper and better to use the natural meal than any of the commercial enzyme preparations. It is not even necessary to prepare an extract. The amount required is about 0.3 gm., which can be approximately measured by means of a small spatula, tube or spoon after a few trials. It is important to use a fine meal.\* It will be found that the natural beans are rather difficult to grind finely in a coffee mill.

Some samples of meal yield a trace of ammonia, when treated with potassium carbonate, but in the author's experience this is usually so small as to be outside the error of experiment and can be neglected.

*Method.* See that the tube B (fig. 44) and the narrow tube that goes into it have been well washed, and are quite free from any of the alkaline carbonate used in a previous experiment.

(i.) Measure 0.5 cc. of the urine into B, using an accurate Ostwald pipette (fig. 51). If the urine is known to be a very dilute one, 1 or even 2 cc. can be taken.

(ii.) Add 2 cc. of the acid potassium phosphate, and 3 cc. of water, washing the traces of urine down to the bottom of the tube with these two fluids. Then add 0.3 to 0.4 gm. of the Soya bean meal. Lightly shake to mix.

(iii.) Add 2 drops of caprylic alcohol, to prevent subsequent foaming.

(iv.) Fit the rubber stopper with the tubes it carries.

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\* Soya bean meal can be obtained from Messrs. Baird and Tatlock, London.

(v.) Into E (or the flask shewn in fig. 45) measure 20 cc. of the standard sulphuric acid add 2 drops of caprylic alcohol, fit the stopper and connect up E to the pump and B to the wash bottle A.

(vi.) Immerse the tube B in a beaker or can of water at a temperature of about 45° C., and leave it for 12 minutes, a slow current of air should be drawn through the apparatus to ensure a thorough mixing of the contents and extraction of the enzyme from the meal.

(vii.) Remove the tube from the bath and send a strong air current through the apparatus for 1 minute to sweep over any ammonia that may have escaped from the fluid and be present in the air of B.

(viii.) Stop the air current and disconnect the entry and exit tubes of B. Remove the stopper with these tubes and place it on the bench in such a way that the small amount of fluid on the end of the entry tube is not lost. Cool the tube B. in a stream of cold water.

(ix.) Add 4 to 5 grams. of pure dry potassium carbonate to B, roughly measuring it with a suitable spoon, immediately replace the stopper and connect up the apparatus. Send a slow air current through for 2 minutes and then a rapid current for 12 minutes.

(x.) Proceed as described in Ex. 399 (iv.).

*Calculation.* Determine the percentage of nitrogen in the form of ammonia and urea as described for Kjeldahl's method, p. 323. The result thus obtained is the mgms. of (urea + ammonia) N. in 0.5 cc. urine = Ua.

So mgms. of (urea + ammonia) N. in 100 cc. =  $200 \times Ua$ .

From Ex. 399, the mgms. of ammonia N. in 100 cc. = 20 A.

So mgms. of urea N. in 100 cc. =  $(200 \times Ua) - 20 A = U$ .

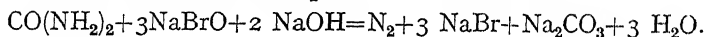
Since 60 grams. of urea contain 28 grams. of nitrogen, the grams. of urea in

$$100 \text{ cc. urine} = U \times \frac{60}{28} \times \frac{1}{1000} = U \times 0.00214 \text{ (log. } \bar{3}.3310).$$

#### 401A. The estimation of urea by the hypobromite method.

*Principle.* Urine is treated with an alkaline solution of sodium hypobromite and the amount of urea calculated from the volume of nitrogen evolved.

The reaction that takes place is as follows:—



Hence 60 grams urea evolve 28 grams N. =  $2 \times 11.2$  litres. Therefore 1 gram urea evolves 373 cc. Nitrogen, measured at N.T.P.

Practically it is found that only 357 cc. are evolved, the other 4.4 per cent. of the nitrogen being converted into nitrates, cyanates, etc.

*Apparatus.* See fig. 46. A 50 cc. burette (*a*) is held by a clamp in a tall cylinder of water (*b*). The upper end of the burette is closed by a tightly-fitting rubber stopper, which is pierced by one limb of a glass T-piece. The upper limb of the T-piece is fitted with a short length of pressure-tubing carrying a screw-clamp (*e*). The side limb of the T-piece is connected by about two feet of small rubber tubing to a glass tube piercing the well-fitting rubber stopper of a wide-mouthed bottle (*c*) of about 60 cc. capacity. This bottle is placed in a jar of water, supported at such a height that the burette can be lifted nearly out of the tall cylinder without stretching the rubber connexion. A small glass bottle or short tube of 10 to 15 cc. capacity is also required (*d*). (For the method of preparing the hypobromite solution see p. 395.)

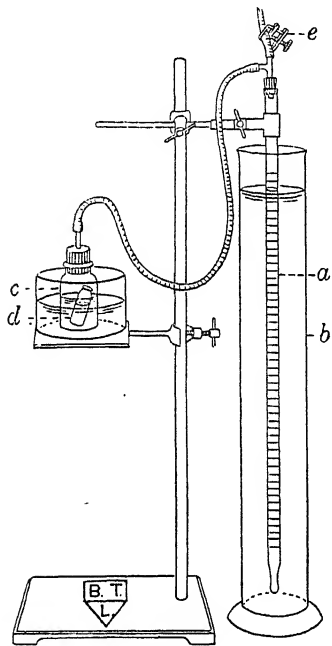


Fig. 46. Apparatus for determination of urea by hypobromite method.

*Method of Analysis.* Place 25 cc. of freshly-prepared hypobromite solution in (*c*). Put 4 cc. of urine, accurately measured, in the small bottle (*d*), and place this inside the other by means of a pair of forceps, taking great care not to upset any urine into the hypobromite. Fit the rubber cork tightly into the bottle and place this in the jar of water to cool. See that the burette is as low as possible, that the cylinder has sufficient water in it to reach the zero graduation of the burette, and that the screw clamp is open. Leave the apparatus to cool to the temperature of the water; clamp the burette in such a position that the water is below the zero mark, and then screw the clamp on the rubber tubing as tight as possible. Note down the level of the water in the burette, keeping the eye level with the meniscus. Take the bottle out of the jar, and gently tilt it so that the urine flows into the hypobromite.

Gently shake the bottle from side to side, keeping the bottle upright to prevent the froth from being forced up into the tube. Tilt the bottle again and repeat the process till the urine and hypobromite are thoroughly mixed. Place the bottle back in the jar of water for about 3 minutes to cool. Raise the burette till the level of water in the tube is the same as that outside, the gas being thus under atmospheric pressure. Read the level of the meniscus as before : the difference in the two readings is the volume of nitrogen evolved. Ascertain the temperature of the water and the barometric pressure.

*Calculation of results.*

Let the temperature be  $t^{\circ}$  C., the tension of aqueous vapour at this temperature be  $T$  mm. (see Appendix), and the barometric pressure be  $B$  mm. of mercury. Let  $v$  be the volume of nitrogen measured under the conditions : at  $0^{\circ}$  C. and 760 mm. this will become

$$\frac{v \times 273 \times (B - T)}{(273 + t) \times 760} = v'$$

Now 357 cc. of N are evolved from 1 gram of urea.

$\therefore v'$  cc. are evolved from  $\frac{v'}{357}$  gram of urea.

$\therefore$  4 cc. urine contain  $\frac{v'}{357}$  gram urea.

and 100 cc. urine contain  $\frac{25v'}{357}$  gram urea.

NOTE.—Performing these two calculations in one operation we obtain for the percentage of urea

$$\frac{v \times (B - T)}{(273 + t)} \times \frac{273 \times 25}{760 \times 357} = \frac{v \times (B - T)}{(273 + t)} \times .02515 \text{ (log. } \bar{2}.4006).$$

### E. Creatinine and Creatine.

The methods that are universally adopted are based on Jaffe's test for creatinine (Ex. 227) as applied by Folin for colorimetric estimation. Creatine does not reduce picric acid, but is converted to creatinine either by heating with hydrochloric or picric acid. The combined (creatinine + creatine) is then estimated colorimetrically. Folin and Doisy\* have recently pointed out that considerable errors may occur if impure picric acid is used, especially in

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\* Folin and Doisy, *Journ. Biol. Chem.*, xxviii., p. 349.

the case of the estimation of creatine. They give a method of purifying the very doubtful specimens of wet picric acid that are at present on the market. A much less troublesome method is given on p. 251, but for observations on the excretion of creatine in pathological conditions it would be safer to follow Folin.

Graham and Poulton\* have shown that the presence of aceto-acetic acid in the urine inhibits the reaction with creatinine, so that the estimations are too low. This acid is destroyed by the heating required for the estimation of (creatinine + creatine), so that the result of the analysis always makes it appear as if creatine were present. They give a method for the removal of aceto-acetic acid, which must be followed when the urine gives a distinct Rothera's test. They are unable to confirm the statement that creatine is found in the urine as a result of carbohydrate starvation. It only appears to be present if faulty analytical procedures are adopted, aceto-acetic acid always being found in carbohydrate starvation.

#### 402. The estimation of creatinine (Folin).†

*Principle.* A measured amount of the urine is treated with picric acid and caustic soda. The picric acid is reduced to picramic acid in the cold by the creatinine present, glucose having no effect in the cold (see Exs. 108 and 227). A known amount of creatinine is similarly treated and the solutions compared in a colorimeter.

##### *Solutions and apparatus required.*

1. A colorimeter, see p. 384.
2. Standard solution of creatinine zinc chloride. 1.6106 gram. of the pure recrystallised zinc compound (see p. 300) is dissolved in about 500 cc. of distilled water and 100 cc. of Normal hydrochloric acid and the volume made up to 1 litre with distilled water. 1 cc. contains 1 mgm. of creatinine. The solution is quite stable.
3. A saturated aqueous solution of *pure* picric acid (about 1.2 per cent.) and a 20 cc. pipette for measuring it.
4. 10 per cent. caustic soda, which can be measured by a pipette or burette.
5. Ostwald pipettes (fig. 51) of 1 cc.
6. Two 100 cc. measuring flasks.

*Method.* Into a 100 cc. measuring flask (labelled "U") measure 1 cc. of the urine by means of an Ostwald pipette. Add 20 cc. of the picric acid and then 1.5 cc. of the soda. Allow the mixture to stand for 10 minutes with gentle agitation. As soon as the mixture has been made measure 1 cc. of the standard creatinine solution into the other 100 cc. flask (labelled "S"), add the picric

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\* Graham and Poulton, *Proc. Roy. Soc.*, lxxxvii., B., p. 205.

† *Journ. Biol. Chem.*, xvii., p. 470.

acid and soda as before and mix, noting the time. After the flasks have each stood for 10 minutes they are separately filled to the mark with distilled water and the contents well mixed. The solutions are then compared in a colorimeter (see p. 388), the standard being set at 15 mm.

Should "U" read below 10 mm., the determination must be repeated, using 1 cc. of an accurately diluted urine, say 1 in 2 or 1 in 3. Should "U" read above 22 mm. the determination must be repeated with 2 cc. or more of the urine. In such cases there is no necessity to make another standard, the colour being quite permanent for hours.

*Calculation.*

$$\frac{\text{Mg. in 1 cc. urine}}{\text{Mg. in 1 cc. standard}} = \frac{\text{Reading of "S" }}{\text{Reading of "U" }} = \frac{15}{\text{Reading of "U" }}$$

$$\text{Mg. in 1 cc.} = \frac{15}{\text{Reading of "U" }} = \text{Cn.}$$

If more or less than 1 cc. of urine have been taken, this must be divided by the volume of urine used.

Grams. of creatinine in 100 cc. = Cn  $\times$  0.1.

Since 113 grams. of creatinine contain 42 grams. of nitrogen, grams. of creatinine- N in 100 cc. = Cn  $\times$  0.1  $\times$   $\frac{42}{113}$  = Cn  $\times$  0.371 (log. 2.5701).

**403. The estimation of creatine and creatinine (Folin).**

- (i.) Weigh a 200 cc. Erlenmeyer flask of "Duro" glass.
- (ii.) Into it measure the amount of urine that contains between 0.7 and 1.5 mgm. of creatinine, as determined by the previous exercise.
- (iii.) Add 20 cc. of saturated picric acid and about 130 cc. of water and a few pieces of broken porcelain.
- (iv.) Boil gently over a micro-burner for 1 hour.
- (v.) Increase the heat and boil down to rather less than 20 cc.
- (vi.) Weigh the flask and add water, if necessary, to make the total weight of the contents equal to 20-25 grams.
- (vii.) Cool in running water.



(viii.) Add 1.5 cc. of 10 per cent. soda from a burette and allow the mixture to stand for 10 minutes with gentle agitation.

(ix.) Transfer to a 100 cc. volumetric flask and wash out with distilled water to make 100 cc.

(x.) Estimate colorimetrically as in the previous exercise.

*Calculation.* This is the same as in the previous exercise, proper allowance being made for the volume of urine used. The difference between the two results is the creatine, which is usually expressed in terms of creatinine. To convert this to creatine it should be multiplied by

$$\frac{129}{113} = 1.141 \text{ (log. } .0575\text{)}.$$

#### 404. The estimation of creatine and creatinine (Benedict).\*

*Principle.* The dehydration of creatine to creatinine is very rapidly effected by evaporation to dryness with hydrochloric acid. A little lead is added to inhibit pigment formation, the traces of hydrogen evolved preventing oxidation. It is not applicable to urines containing glucose.

*Method.* Into a small beaker measure that volume of urine that contains 7 to 10 mgm. of creatinine. Add 10 to 20 cc. of N. hydrochloric acid and a pinch or two of powdered or granulated lead. Boil down over a small free flame till nearly dry and then evaporate to *complete* dryness on a boiling water bath. Add 10 cc. of hot distilled water and filter through a small plug of cotton wool into a narrow 25 cc. measuring cylinder. Wash out quantitatively with two successive portions of about 4 cc. of hot water. Cool by immersion in cold water and make the volume up to 20 cc. Measure 2 cc. into a 100 cc. volumetric flask, using an Ostwald pipette. Add 20 cc. of saturated picric acid and 1.5 cc. of a 10 per cent. solution of soda that contains 5 per cent. of Rochelle salt (to prevent the formation of a cloud due to traces of dissolved lead). After standing for 10 minutes with gentle agitation, make up to the mark with distilled water. A standard is simultaneously prepared from 1 cc. of the standard creatinine solution, 20 cc. of picric acid and 1.5 cc. of the 10 per cent. soda containing 5 per cent. of Rochelle salt, diluted to 100 cc. after standing for 10 minutes. The two solutions are read as described in Ex. 402.

\* *Journ. Biol. Chem.*, xviii., p. 191.

*Calculation.* If (*a*) cc. of urine have been taken originally, the amount actually used corresponds to  $\frac{(a)}{10}$ . If the standard is set at 15 mm., and the "U" tube reads at U, then mg. of (creatinine + creatinine) in

$$1 \text{ cc.} = \frac{15 \times 10}{U \times (a)}.$$

The rest of the calculation is the same as that of the previous exercise.

NOTE.—The above method is a slight modification of that published by Benedict, but it does not differ in any essential.

#### 405. The removal of aceto-acetic acid (Graham and Poulton).

*Principle.* Aceto-acetic acid is converted by heat to acetone, which is distilled off at low pressure. The following account is slightly modified from the original.

##### *Solutions and apparatus required.*

- (i.) A 10 per cent. solution of phosphoric acid.
- (ii.) A solution of soda of such a strength that 1 cc. of it neutralises 1 cc. of the phosphoric acid, phenol phthalein being used as the indicator. A 15 per cent. solution of soda is a convenient starting point for the preparation of this. When it is correctly adjusted, 1.5 cc. of it will neutralise all three valencies of 1 cc. of the phosphoric acid, only two of which are neutralised to phenol phthalein.
- (iii.) A suction pump and gauge (see fig. 9, p. 74).
- (iv.) A thick walled tube (25 to 30 mm. by 200 mm.) similar to the tube E of fig. 44, but in place of F is substituted a tube drawn out to a fine capillary, which must reach nearly to the bottom of E. (The upper end of the tube may be fitted with a piece of pressure tubing and a screw clip similar to that shown in C of fig. 8.)

*Method.* Into the tube measure 10 cc. of the urine and add 1 cc. of the 10 per cent. phosphoric acid. Fit the stopper carrying the capillary tube and connect the other outlet tube to the tube E of the apparatus shewn in fig. 9 and have C turned to make connexion with A. Turn on the pump and note the pressure obtained, which depends on the size of the capillary, on the size of A and on the water pressure. It is necessary to maintain a pressure of about 210 mm. of mercury. Immerse the tube containing the acidified urine in a water bath kept between 65° and 70° C., and leave it for about three-quarters of an hour, seeing that the temperature does not rise above 70° C., nor the pressure fall below 210 mm. of mercury. Release the pressure by turning the tap C to connect with B, and then turn off the water. Disconnect and cool the solution under the tap. Add 1.5 cc. of the standardised soda to completely neutralise

the phosphoric acid and then transfer to a narrow 25 cc. cylinder. Wash out with water to make a total volume of 20 cc. Mix well and estimate the creatinine by the method given in Ex. 402. 2 cc. of the solution correspond to 1 cc. of the urine.

### F. Uric Acid.

Most of the methods employed at present are based on one of two main principles. The first is on Hopkins' ammonium chloride method; the other is colorimetric with Folin's reagent. The author's experience with all modifications of the latter has been so unfavourable that it has been reluctantly abandoned. It is possible that the difficulty of obtaining reliable chemicals accounts for many of the troubles, but the greatest care in this respect has not been rewarded with success.

Hopkins' is the standard method. It requires skill and practice to get good results, but it is absolutely reliable. The modification of it introduced by Folin and Schaffer is a concession to the unskilful manipulator, but it has the disadvantage of an allowance of 3 mgms. for the ammonium urate not precipitated by ammonium sulphate. The author humbly suggests that this is an averaging of results, for comparisons with Hopkins' original method and also with the modification described below, seem to indicate that with certain specimens of urine the allowance should be smaller or greater than this. It is always the same for a given specimen of urine, suggesting that some unknown factor affects the solubility of ammonium urate under the conditions of the experiment.

It is an objection to Hopkins' method that the result cannot be obtained rapidly, as the solution must be allowed to stand over-night for the whole of the uric acid to crystallise out. This inconvenience is also a feature of the Folin-Schaffer modification. Many attempts have been made to titrate the original precipitate of ammonium urate, but they have not been very successful owing to the difficulty of removing the chlorides which also titrate with the permanganate in acid solution. It is generally stated that the addition of manganese sulphate prevents the action of the chlorides. By accidentally using magnesium sulphate on one occasion the author was led to investigate carefully the extent to which the presence of chlorides interfere with a correct result. It was found that moderate concentrations have no effect, owing to the great rate at which uric acid is oxidised compared to the low velocity of the reaction between chlorides and permanganate. As a result of a considerable amount of work the modification described below was elaborated. The final result can be obtained in  $1\frac{1}{2}$  hours, and in the hands of the author agrees to 1 mgm. per 100 cc. with that of Hopkins' original method. It has been regularly used in class work for the past 4 years, and presents little difficulty to the average student. But it must be admitted that it has not been tested with a large number of pathological urines, and for that reason it is possible that in certain cases it will only yield approximate results. There is no *a priori* reason why it should fail more than other methods.

#### 406. Uric acid (Cole's modification of Hopkins' method).

*Principle.* The urine is treated with colloidal iron to remove an unknown substance that is precipitated by ammonium chloride. The filtrate is treated with solid ammonium chloride and made strongly alkaline with ammonia.

The uric acid is rapidly and quantitatively precipitated as ammonium urate. This is filtered off, washed with ammonium sulphate to remove the greater part of the chlorides, dissolved in hot sulphuric acid and titrated with standard potassium permanganate. The end point is reached when a momentary pink flush is seen over the whole body of the fluid. This marks a stage when the rate of oxidation of the uric acid suddenly decreases. Up to this point 1 cc. of 0.05 N. permanganate is found empirically to correspond to 3.7 mg. of uric acid. The chemical changes involved in the oxidation have not yet been determined.

*Solutions and reagents required.*

- (i.) Colloidal (dialysed) iron, 0.6 per cent.
- (ii.) Pure, dry, recrystallised ammonium chloride.
- (iii.) Washing fluid. 100 grams. of pure ammonium sulphate are dissolved in about 800 cc. of distilled water, 10 cc. of strong ammonia are added and the volume made up to 1 litre with water. It is convenient to use this from a wash bottle with a fine jet.
- (iv.) 45 per cent. sulphuric acid (by volume). To 500 cc. of distilled water in a large flask cautiously add 450 cc. of pure concentrated sulphuric acid, cooling at intervals. Cool thoroughly under the tap and make up the volume to 1,000 cc.
- (v.) 0.05 N. potassium permanganate. Dissolve 1.58 gram. of the pure salt in distilled water and make the volume up to 1,000 cc. Care must be taken to see that the whole of the solid has dissolved before the solution is used. It can be titrated against pure ammonium oxalate as described on p. 132. If  $Ox.$  be the weight of the oxalate taken in grams. (about 0.15) and  $P$  the volume of permanganate

required, then the normality is  $\frac{Ox.}{P \times 0.07105} = P_n$ .

1 cc. of 0.05 N. permanganate  $\equiv$  3.7 mgms. uric acid.

1 cc. of  $P_n$ N. permanganate  $\equiv \frac{3.7 \times P_n}{0.05}$  mgms.

*Method.* Measure 150 cc. of the urine into a 200 cc. beaker, marking the 100 cc. level by means of a label. Add 30 cc. of the colloidal iron, stirring well during the addition. Filter through two dry papers into two dry flasks (two being used to save time). When at least 100 cc. of the filtrate has been collected, carefully measure the amount taken and transfer it to the marked beaker, which has been previously washed and drained. It is convenient to take 100 cc. but with dilute urines it is better to take 120 or 150 cc. For every 10 cc. of the filtrate taken weigh out 2 grams. of the solid ammonium chloride; add this to the beaker and stir well. When it has dissolved add 3 cc. of concentrated ammonia and stir again. Stir at intervals for 20 minutes, then remove the rod and let it rest on the rim of the beaker. When the bulk of the precipitate has settled

the urate is filtered off either through a plain paper, or more rapidly by moderate suction through a paper and paper pulp supported on a perforated plate (20 to 25 mm. diameter) resting in a funnel, as shewn in the accompanying figure. The filter is prepared by cutting a piece of filter paper rather larger than the disc, placing this in position, moistening it and applying suction by a pump. Any creases round the edge are flattened out by the point of a pencil.

The pressure being released a *little* paper pulp is poured on to the disc and allowed to settle and then gentle suction applied. The pulp will completely seal the cracks between the disc and the funnel. It is advisable to cut another circle of paper, smaller than the first, and to place it on the centre of the pulp. It prevents the latter being washed away during filtration. Filter the supernatant fluid first, taking care not to disturb the bulk of the precipitate. Do not

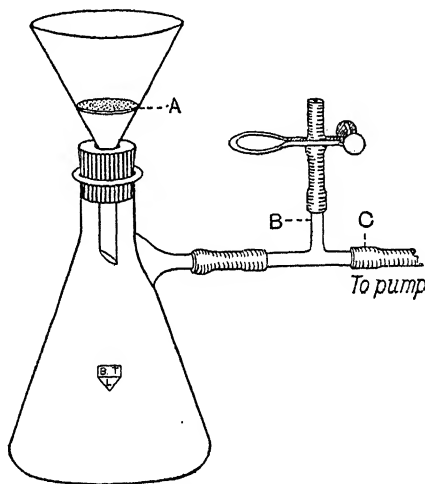


Fig. 47.

use too high a pressure, as this drives the amorphous ammonium urate into a cake which renders the subsequent washing very slow. The pressure can be regulated by use of the apparatus shewn on page 74. If this is not available, a T-piece can be used, as shewn in fig. 47. One limb of this is connected to the pump and the other is fitted with a piece of pressure tubing and a spring clip, by means of which the pressure can be instantaneously released. When the main mass of the fluid has passed through, transfer the bulk of the precipitate, but do not suck quite dry. Wash out the precipitate remaining in the beaker with the ammonium sulphate solution on to the filter and start suction again. Do this twice more, finally sucking the precipitate dry. The object of the washing is to remove as much ammonium chloride as possible from the precipitate, paper

and beaker. Now transfer the paper, precipitate and disc to the marked beaker, by means of a glass rod which has a fine curved point. Remove the funnel from the filtering flask and wash it down into the beaker with a jet of hot water. Also wash the pointed glass rod. Add hot water to make a total volume of 100 cc., as indicated by the label. Add 20 cc. of the 45 per cent. sulphuric acid and stir with a thermometer. The whole of the precipitate must be dissolved, if necessary by the aid of heat, before the titration is commenced. Cool or heat to 65° C. Titrate with the standard permanganate, reading the meniscus by the aid of a lighted match held behind the burette. The permanganate must be added rather slowly, with constant stirring. The end point is reached when the addition of a couple of drops causes a *faint* pink flush through the whole body of the fluid. A very considerable addition has to be made for the pink to be permanent, but the empirical valuation of the permanganate is based on the end point described above.

*Calculation.* Since 150 cc. of the urine were treated with 30 cc. (one-fifth volume) of colloidal iron, 6 cc. of the filtrate from this correspond to 5 cc. of urine.

If  $u$  cc. of the filtrate have been taken and  $p$  cc. of 0.05 N. permanganate used, then  $u$  cc. contain  $p \times 3.7$  mgms. uric acid, and 100 cc. of urine contain  $p \times 3.7 \times \frac{6}{5} \times \frac{100}{u} \times \frac{1}{1000}$  gram. =  $\frac{p}{u} \times 0.444$  gram.

NOTE.—Another method of filtering off the urate is to use a Hirsch funnel with a fixed perforated plate, and to use asbestos instead of paper pulp. The filtering mat is prepared in the way described for a Gooch crucible (see p. 393), the drying being omitted. The mat with the layer of urates can be very neatly removed, and the whole process is much facilitated. The time required for the estimation is considerably overstated above. The whole process can be accomplished in 40 minutes, it being unnecessary to allow the fluid to stand for more than 10 minutes after the addition of the ammonia.

## G. Glucose.

The estimation of sugar in urine when there is a considerable amount present can be carried out by any of the standard methods, as, owing to the dilution necessary or the small amount required, there is little interference by the normal urinary constituents. If the concentration is less than 0.8 per cent. the author prefers to use the Wood-Ost method (p. 131), and to dilute the urine at least three times with water. Though the amount of permanganate required is small, the results are quite satisfactory. With Benedict's method of direct titration (p. 127) the end point is apt to be very uncertain with low concentrations of sugar. The polarimetric method referred to on

page 127 is of great service when a large number of diabetic urines have to be examined. The results may be rather low owing to the presence of the laevorotatory  $\beta$ -oxy-butyric acid.

The method described below is the only one at present available for the estimation of such small amounts of glucose as are present in normal urine. It is included in the hope that the study of slight variations from the normal will extend our knowledge of the pathology of diabetes, and also as a practical method for the detection of lowered tolerance to carbohydrates.

#### 407. The estimation of glucose in normal urine (Benedict and Osterberg).\*

*Principle.* The urine is treated with mercuric nitrate and neutralised with sodium bicarbonate. The creatinine, urates, etc., are thus removed. The mercury is removed by means of zinc and the sugar estimated by the colorimetric method with picric acid.

##### *Solutions and apparatus required.†*

1. *Picric-picrate mixture*, see p. 251.

2. *Standard solution of glucose.* The stock solution is described on p. 251. 5 cc. of this are diluted to make 50 cc. with distilled water. 1 cc. contains 1 mg. glucose.

An alternative standard can be prepared from pure picramic acid. The stock solution is described on p. 251. 105 cc. of this are treated with 0.5 cc. of 20 per cent. sodium carbonate and 15 cc. of the picric-picrate mixture and diluted to make 300 cc. with distilled water. The colour obtained corresponds to that of 1 mg. glucose in 4 cc. of water, treated as described for the final urine filtrates and the coloured solution diluted to 25 cc. It is the most convenient standard to use, as time is saved and a possible error of measurement avoided.

3. *Sodium carbonate solution*, 20 per cent., see p. 252.

4. *Test-tubes graduated at 12.5 and 25 cc.*, see p. 252.

5. *Ostwald pipettes*, see p. 385.

6. *A colorimeter*, see p. 388.

7. *Mercuric nitrate solution*, see A solution, p. 395. On no account must the B solution be used as a substitute.

*Method.* Into a 50 cc. beaker measure 20 cc. of the urine and then 20 cc. of the mercuric nitrate solution. Mix and add solid sodium bicarbonate with gentle shaking. Considerable frothing occurs. The bicarbonate can be added fairly freely until this ceases. Stir well and see that the material on the sides of the beaker is mixed with the main mass, which forms a kind of paste. Now add the bicarbonate until the fluid reacts just alkaline to litmus paper. Filter at once through a dry paper into a small dry

\* *Journ. Biol. Chem.*, xxxiv., p. 195.

† These can be obtained from Messrs. Baird and Tatlock, London.

flask. The filtrate should be quite clear and colourless. Add a pinch of zinc dust and 2 drops of concentrated hydrochloric acid, shake and allow it to stand for 5 to 10 minutes. Filter through a small dry filter into a dry test-tube.

Measure 1 to 4 cc. of this filtrate (so that 0.5 to 2 mg. sugar are taken) into one of the graduated tubes. If less than 4 cc. are taken make the volume up to exactly 4 cc. with distilled water. Add 1 cc. of the 20 per cent. sodium carbonate and 4 cc. of the picric-picric mixture and plug the tube with cotton wool. If the standard is prepared from glucose, measure 1 cc. (*i.e.* 1 mg.) into another graduated tube (marked "S"). Add 3 cc. of water, 1 cc. of the sodium carbonate, 4 cc. of the picric-picric mixture and plug with cotton wool. Immerse both tubes in a boiling water bath and note the time. After exactly 10 minutes remove the tubes and cool thoroughly under the tap. Dilute the "S" tube to 25 cc. If picramic acid is used as a standard, fill a tube with some of the dilute solution. Dilute the other tube to 12.5 cc. or to 25 cc. depending on the colour obtained. If on diluting to 25 c.c. the colour is still much darker than the standard, the experiment must be repeated, using less of the final filtrate from the zinc or diluting the urine and starting again from the beginning.

When the two colours are roughly the same, compare with the standard in a colorimeter (see p. 388), setting the standard at a height of 15 mm. It may be necessary to filter the solution containing the urine from a slight precipitate that appears on heating with the alkali and picrate.

*Calculation.* This depends on the amount of final filtrate taken and on the dilution. Suppose that 2 cc. of final filtrate were taken and it was diluted to 12.5 cc., and that the reading was 17.4 mm., against the standard at 15. Now 2 cc. of the filtrate contain 1 cc. of urine, and the standard contains 1 mg. of glucose (or corresponds to this if picramic acid be used). Since the urinary solution was only diluted to 12.5 cc., whilst the standard was made up to 25 cc., the result must be halved.

$$\text{So} \quad \frac{\text{mg. of glucose in 1 cc.}}{1} = \frac{15}{17.4} \times \frac{1}{2}.$$

In general

$$\begin{aligned} \text{mg. of glucose in 1 cc.} &= \frac{\text{Reading of "S"}}{\text{Reading of "U"}} \times \frac{\text{Volume after heating}}{\text{Volume filtrate used}} \times \frac{2}{25} \\ &= \frac{15}{17.4} \times \frac{12.5}{2} \times \frac{2}{25}. \end{aligned}$$



NOTE.—The above method gives the total of glucose and non-fermentable carbohydrate. Benedict and Osterberg give the following for the determination of the latter. To 25 cc. of urine (free from preservative) in a cylinder or test-tube add 20 to 25 mg. of glucose and about one-quarter cake of yeast. Mix well and allow to stand in the incubator at 35-38° C. for 18 to 20 hours. Decant 15 to 20 cc. of the urine and determine sugar as before fermentation. The difference between the two gives the fermentable sugar.

## H. The Acetone Bodies.

A very large number of methods have been proposed, and in this case the latest is undoubtedly the best, since Van Slyke's method gives the  $\beta$ -oxybutyric acid as well as the acetone and aceto-acetic acid. Since the  $\beta$ -oxybutyric acid usually forms about 75 per cent. of the total acetone bodies excreted, its estimation is of the utmost importance in all studies related to the origin and excretion of these substances. The method given in Ex. 411 is that used by the author for the determination of acetone and aceto-acetic acid when a large number of cases have to be studied. It is the most rapid method of obtaining a good indication of the severity of the ketosis.

### 408. Total acetone bodies (D. Van Slyke).\*

*Principle.* The urine is treated with copper sulphate and lime to remove sugar and other interfering substances. The filtrate is boiled with mercuric sulphate and sulphuric acid under an inverted condenser. Potassium dichromate is run in down the condenser to oxidise the oxy-butyric acid to acetone, whilst the aceto-acetic acid is very rapidly converted to acetone by the influence of the hot acid. The acetone forms an insoluble compound with mercury which is filtered off and weighed in a Gooch crucible. The precipitate can be titrated if desired by a method described in the original paper.

#### *Solutions required.*

1. *Copper sulphate.* 200 grams. of the pure crystalline salt are dissolved in water and made up to 1 litre.
2. *Mercuric sulphate solution.* 73 grams. of pure red mercuric oxide are dissolved in 1 litre of 4 N. sulphuric acid.
3. *Sulphuric acid.* To 500 cc. of distilled water in a large flask cautiously add 500 cc. of concentrated sulphuric acid. Cool thoroughly under the tap and make up to 1 litre with distilled water. Titrate a portion of 2 cc. with N. soda (or titrate 5 cc. with 5 N. soda) and adjust to 17 N. if necessary.
4. *Calcium hydroxide suspension.* Mix 100 grams. of pure "light" calcium hydroxide with 1 litre of distilled water.
5. *Potassium dichromate.* Dissolve 50 grams. in water and make up to 1 litre.

*Method.* (i.) Measure 25 cc. of the urine into a 250 cc. volumetric flask. Add 100 cc. of distilled water, 50 cc. of the copper sulphate solution and mix. Then add 50 cc. of the calcium hydroxide suspension (previously well shaken) and shake well. Test the

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\* *Journ. Biol. Chem.*, xxxii., p. 455.

reaction with litmus. If not alkaline add more of the calcium hydroxide. Dilute to the mark and allow it to stand for at least 30 minutes. Filter through a dry folded paper into a dry flask. This will remove up to 8 per cent. of glucose. If more than this is present, the urine must be diluted to bring it down to 8 per cent. If glucose is present in the filtrate a *yellow* (not white) precipitate will appear if a little of it is boiled in a test-tube.

(ii.) Connect up a 500 cc. Erlenmeyer flask with a straight reflux condenser, as shewn on p. 72. Into the flask measure 25 cc. of the urine filtrate, 100 cc. of water, 10 cc. of the 17 N. sulphuric acid and 35 cc. of the mercuric sulphate solution. Connect up to the condenser and heat to boiling over a free flame (not over a sand bath). When boiling has begun add 5 cc. of the dichromate solution, running this down the condenser tube. Allow the mixture to boil gently for  $1\frac{1}{2}$  hours.

(iii.) Cool the solution and filter off the precipitate, using a weighed Gooch crucible (see notes to Ex. 410). Wash out the flask with cold water, of which about 200 cc. in all should be used. Dry the crucible in an hot-air oven at  $110^{\circ}$  C. for an hour. Allow the crucible to cool down to air temperature and weigh again.

*Calculation.* If 25 cc. of the filtrate (representing 2.5 cc. of the urine) are used, 1 gram. of precipitate corresponds to 2.48 grams. of total acetone bodies per 100 cc. in terms of acetone. This is on the assumption that the molecular proportion of the acetone bodies in the form of  $\beta$ -oxy-butyric acid is 75 per cent., the usual figure.

409.  **$\beta$ -oxy-butyric acid.** The acetone and aceto-acetic acid are first boiled off and then the estimation conducted as before. Measure 25 cc. of the filtrate into the open flask, add 100 cc. of water and 2 cc. of the sulphuric acid. Boil gently for 10 minutes with a free flame. Cool and transfer to a measuring cylinder and note the volume. Return it to the flask and add water to the cylinder to make a total volume of 127 cc. Add 8 cc. of the sulphuric acid and 35 cc. of the mercuric sulphate. Connect up to the condenser and boil. When boiling add 5 cc. of the dichromate and allow the mixture to boil gently for  $1\frac{1}{2}$  hours. Then proceed as in Ex. 408 (iii).

*Calculation.* 1 gram. of precipitate corresponds to 2.64 grams. of  $\beta$ -oxy-butyric acid per 100 cc., reckoned as acetone: to convert to  $\beta$ -oxy-butyric acid multiply by  $\frac{104}{58} = 1.793$ .

410. **Acetone and aceto-acetic acid.** This can be found by difference between the two previous exercises, or it can be determined separately by the method adopted for total acetone bodies, except that (1) no dichromate is added, and (2) the boiling is continued for not less than 30 nor more than 45 minutes.

*Calculation.* 1 gram. of precipitate corresponds to 2.0 grams. of acetone and aceto-acetic acid per cent., reckoned as acetone.

NOTES.—1. Van Slyke also gives a method of titrating the mercury precipitate. It is presumably quicker, since it is not necessary to prepare and dry the Gooch crucible. The author has no experience of it.

2. The Gooch crucible should be of 25 to 50 cc. capacity, and fitted as shewn in fig. 48. The asbestos mat can be prepared as described on page 393. It is thoroughly washed and firmly sucked down and dried in a steam oven or a hot-air oven at 110° C. It is allowed to cool in the air and weighed. It is then fitted to the rubber cup and some distilled water carefully added and sucked gently through before the mercury precipitate is filtered off. Several precipitates can be collected and weighed one after another. (See p. 393.)

3. The reagents should be tested by performing an experiment with distilled water instead of urine, starting with the copper sulphate treatment. No precipitate whatever should be obtained. Van Slyke gives a caution that this test should not be omitted.

4. A blank determination of precipitate from other substances in urine other than the acetone bodies may be made by following the procedure of Ex. 409, except that 5 cc. of water are substituted for the dichromate and the boiling period under the condenser is strictly limited to 45 minutes. The weight of precipitate obtained is deducted from that found in any estimation of the acetone bodies. It is usually so small that it can be neglected, except in cases where only small amounts of the acetone bodies are present.

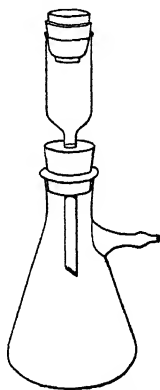
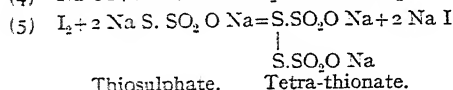
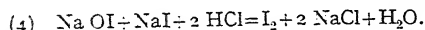
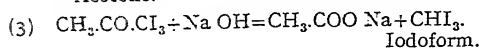
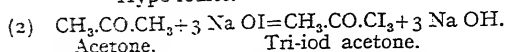
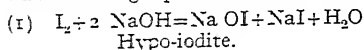


Fig. 48. Gooch crucible and filtering apparatus.

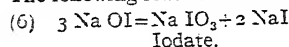
#### 411. The estimation of acetone and aceto-acetic acid by Messinger's method (modified).

*Principle.* The urine is acidified and distilled into a freshly prepared solution of alkaline hypo-iodite, made by adding strong soda to a known volume of standard iodine. The vapours pass through boiling soda which removes any volatile acids that might react with the hypo-iodite. The pre-formed acetone of the urine and that arising from the decomposition of aceto-acetic acid pass over and react with the hypo-iodite to form iodoform. The alkaline solution is then acidified with hydrochloric acid, which liberates any iodine that has not reacted to form iodoform. This excess of iodine is titrated with standard thiosulphate in the usual way. The amount of iodine originally taken being known, and the titration giving the amount that has not reacted with the acetone, the difference is a measure of the amount of acetone distilling over.

The following equations indicate the various reactions that take place :

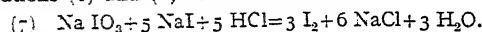


The following reaction also takes place slowly in the alkaline hypo-iodite :



Since iodate does not react with acetone, it is essential to have an excess of at least 10 cc. of the iodine. Further, it is important not to add the iodine to the soda until everything is ready for the distillation.

Reaction (6) does not cause any loss of iodine that would be reckoned as due to acetone, for on acidification the iodate reacts with the iodide shewn in equations (6) and (1) to liberate iodine.



#### Solutions required.

1. *Standard thiosulphate*, about 0.1 N. (See page 254).

2. *Standard iodine*, about 0.1 N. Dissolve 50 gms. of potassium iodide in about 300 cc. of distilled water. Weigh out 27 gms. of iodine, transfer to a large mortar and rub it up with successive portions of the iodide solution until all has dissolved. Wash out with distilled water to make a total volume of 2 litres. Mix thoroughly and make sure that all the iodine has dissolved. Determine the exact strength as follows :

Measure 25 cc. with a pipette into a 250 cc. conical flask. Titrate with the standardised thiosulphate from a burette until the yellow colour of the iodine has nearly disappeared. Add a few drops of soluble starch and complete the titration, as indicated by the complete disappearance of the blue tint.

If  $v$  be the volume of thiosulphate used, and  $t$  its normality, the normality of the iodine is

$$\frac{v \times t}{25} = x.$$

From equation (2) above it will be seen that 1 gm. molecule of acetone reacts with 3 gm. molecules of NaOI, which from (1) is formed from 3  $I_2$ , or 6 litres of normal iodine.

So 6000 cc. N. Iodine  $\equiv$  58 grms. acetone.

1 cc. N. Iodine  $\equiv \frac{58}{6}$  mgrs. acetone.

and 1 cc. of  $x$  N. Iodine  $\equiv \frac{58 \times x}{6}$  mgrs. acetone.

The stock bottle should be labelled with its normality, and the mgms. of acetone equivalent to 1 cc.

Neither the iodine nor the thiosulphate are indefinitely stable. They should both be carefully stored in a cool, dark cupboard, and re-standardised at intervals.

3. *Soluble starch*, 2 per cent. (See page 254).

4. *Strong soda*, about 40 per cent. To 2 litres of distilled water in a large porcelain basin, add 1 lb. of the best powdered caustic soda. Stir well till all has dissolved. Transfer to a flask or Winchester quart bottle, stopper and allow to stand for 24 hours. Syphon off the clear supernatant fluid and store in a bottle stoppered with a rubber cork.

5). *Hydrochloric acid*, pure concentrated.

### Method.

1. *The distillation of the acetone.* Use the apparatus shewn in fig. 49. Into flask A measure an amount of urine that yields between 5 and 15 mg. acetone (10 cc. may be taken for a preliminary trial). Add water to make the volume up to about 50 cc. and then

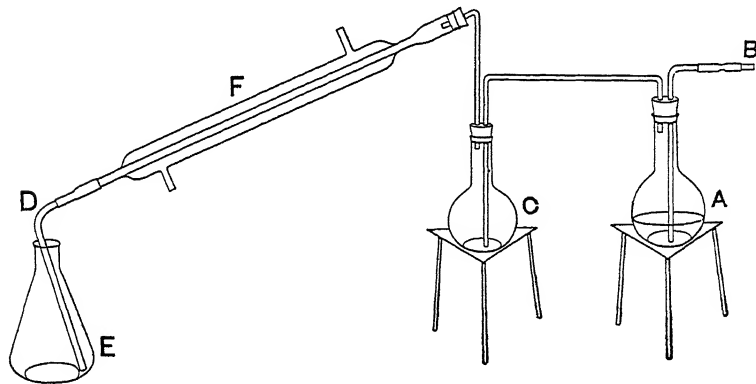


Fig. 49. Apparatus for the estimation of acetone.

A. 300 cc. "Duro" flask. B. Solid glass rod for sealing tube. C. "Duro" flask. D. Glass tube connected by rubber to condenser tube.

E. 250 cc. Erlenmeyer flask. F. Liebig condenser.

1 cc. of strong sulphuric acid. Into flask C place 10 cc. of 40 per cent caustic soda and a few glass beads. Into E place 10 or 20 cc. of 40 per cent. soda and then run in 25 cc. of the standard iodine, measuring this with a pipette. See that the water supply to the condenser is turned on. Close the tube with the glass rod B and then light the burners. The soda in C must boil before the fluid in A. The soda is kept just boiling whilst A is allowed to boil briskly. The first appearance of turbidity in E is noted and the distillation

allowed to proceed for another 10 minutes. Remove plug B and turn out the flames. Detach tube D from the condenser and wash it with a jet of distilled water into E.

2. *Titration of the excess iodine.* Acidify with strong hydrochloric acid, adding this cautiously and cooling well under the tap after the addition of an amount equal to that of the strong soda taken. When the solution is acid, there will be a great intensification of the yellow colour, due to the liberation of iodine. The neutralisation of the alkali makes the solution hot, and if the mixture be acidified before it is cooled a loss of iodine by volatisation will probably occur. An excess of at least 2 or 3 cc. of the acid should be added. Titrate the cold mixture at once with the standard thio-sulphate, running this in until the yellow colour has nearly disappeared. Then add a few drops of the soluble starch and cautiously complete the titration. The end-point is well marked. A return of the blue colour may occur if the reagents employed are not pure, but no notice should be taken of this.

*Calculation and example.*

Thiosulphate was 0.1065 N.

25 cc. iodine required 23.85 cc. of the thiosulphate.

So 1 cc. of thiosulphate  $\equiv \frac{25}{23.85}$  cc. iodine (log. .0204).

And iodine is  $\frac{23.85 \times 0.1065}{25}$  N. = 0.1006 N.

So 1 cc. iodine  $\equiv \frac{23.85 \times 0.1065 \times 58}{25 \times 6}$  mgs. acetone (log. 1.9922).

urine taken = 10 cc.

iodine taken = 25 cc.

Thiosulphate required for back titration = 12.1 cc.

log. of 12.1 = 1.0828

add .0204

anti-log. of 1.1032 = 12.68

25 - 12.68 = 12.32 cc. of iodine used.

log. of 12.32 = 1.0906

add 1.9922

anti-log. of 3.0828 = 12.1.

So 10 cc. urine contain 12.1 mg. acetone.

And 100 cc. urine contain 0.121 gm. acetone from acetone+aceto-acetic acid.

## I. Chlorides.

The usual method for the estimation of chlorides in urine is Volhard's, which is described on page 199 in connexion with the estimation of gastric juice. The method given below is precisely similar. The silver nitrate solution employed is of a different strength and the thiocyanate is not made up to any defined concentration, but standardised against the silver. If preferred the solutions described on page 199 can be employed, 1 cc. of the 0.1 N. silver nitrate being equivalent to 0.00355 gram. of chlorine and 0.00585 gram. of sodium chloride. If this weaker silver solution is used, 25 or 30 cc. of it should be taken for 10 cc. of urine.

A method that is rapid, convenient and accurate, is that of Larrson, but it is now difficult to obtain satisfactory charcoal. Recently, however, the author has been presented with a specimen of charcoal that was prepared for use in gas masks. It seems to be an extremely good adsorbent, superior to the best German products and admirably adapted for all kinds of analytical work. The method is described in the hope that a satisfactory product will soon be on the market. In that case Volhard's method would be superseded by Larrson's.

Van Slyke's method (see page 258) is also well adapted to the estimation of urinary chlorides, especially if only small quantities are available.

### 412. The estimation of chlorides by Volhard's method.

*Principle.* See page 199.

#### Reagents required.

1. Standard silver nitrate solution prepared by dissolving 29.061 grams. of pure fused silver nitrate in distilled water and filling up accurately to one litre. The solution should be kept in the dark.

1 cc. corresponds to .01 gram. NaCl (.00606 gram. Cl).

2. Solution of potassium thiocyanate made by dissolving 8 grams. of the salt in a litre of distilled water.

3. Pure nitric acid, quite free from chlorine.

4. A concentrated solution of iron alum.

*Standardisation of the thiocyanate.* In a beaker place 10 cc. of the silver nitrate, accurately measured: add 5 cc. of pure nitric acid, 5 cc. of iron alum and 80 cc. of distilled water. Titrate the whole with the thiocyanate from a burette until a faint permanent red tinge is obtained. Note the amount required for the 10 cc. of silver nitrate.

*Method.* In a 100 cc. cylinder or measuring flask place 10 cc. of urine, accurately measured by a pipette, 20 cc. of the standard silver solution, also accurately measured, about 4 cc. of pure nitric acid, and 5 cc. of the iron alum. Add distilled water to the 100 cc. mark, and mix thoroughly by pouring into a beaker and stirring well. Filter off the precipitated silver chloride through a dry paper into a dry vessel. Of the filtrate take 50 cc., accurately measured, and titrate it with the potassium thiocyanate solution till a faint permanent red tinge is obtained.

NOTES.—1. It is very important to remember to add the nitric acid. It

renders the silver chloride insoluble and prevents the precipitation of the silver compounds of the purine bases in those cases in which the urine is alkaline.

2. Some workers titrate without filtering off the silver chloride, but the end point is apt to be uncertain owing to the decomposition of the chloride by the thiocyanate.

#### Calculation and Example.

19.6 cc. of the KCNS were required for 10 cc. of the  $\text{AgNO}_3$ .

So 1 cc. of the KCNS  $\equiv \frac{10}{19.6} = 0.51$  cc.  $\text{AgNO}_3$ .

50 cc. urinary filtrate required 11.6 cc. KCNS,

So 100 cc. urinary filtrate would require 23.2 cc. KCNS and would therefore contain  $23.2 \times 0.51 = 11.8$  cc. of the  $\text{AgNO}_3$ .

So  $20 - 11.8 = 8.2$  cc. of the  $\text{AgNO}_3$  have been precipitated.

Now 1 cc. of the  $\text{AgNO}_3 \equiv 0.01$  gm. NaCl,

So NaCl in 10 cc. urine =  $8.2 \times 0.01$  gram.

So NaCl in 100 cc. urine = 0.82 gram.

#### 413. The estimation of chlorides by Larrison's method.\*

*Principle.* The pigments, urates and other interfering substances are removed from the urine by adsorption with charcoal. The chlorides are estimated in a measured amount of the filtrate by direct titration with silver nitrate, using potassium chromate as an indicator.

#### Reagents required.

1. Standard silver nitrate (see Ex. 412).
2. A high quality, pure absorbing charcoal (see p. 394). Ordinary animal charcoal is quite useless.
3. A 5 per cent. solution of potassium chromate.

*Method of analysis.* To 1.5 gram. of the charcoal in a dry 50 cc. flask add 20 cc. of the urine. Shake vigorously and repeat the shaking at intervals for 10 minutes. Filter through a small dry paper into a dry tube. Measure 10 cc. of the filtrate by means of a pipette and transfer it to a small beaker. Add 5 or 6 drops of the chromate and titrate with the standard silver nitrate from a burette until the end point is reached, as indicated by the appearance of a reddish-brown colour.

*Calculation.* 1 cc. of silver = 0.01 gram. NaCl.

*Example.* 10 cc. of the filtered urine required 10.6 cc. of silver.

So 10 cc. contain  $10.6 \times 0.01$  gram. NaCl.

So 100 cc. contain 1.06 gram. NaCl.

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\* *Biochem. Zeitschrift.*, xlix, p. 479.



## 413A. The estimation of chlorides by van Slyke's method.

*Principle, reagents, etc.* (See page 258.)

*Method.* Measure 1 cc. of the urine into a 50 cc. volumetric flask by means of an Ostwald pipette. Add about 25 cc. of distilled water. Then add 10 cc. of the standard silver nitrate by means of a pipette, and then add about 10 cc. of the magnesium sulphate to flock the precipitate. Make up to the mark with distilled water and shake well. Allow to stand for 10 minutes and filter through a dry No. 1 Whatman paper into a dry flask. Measure 25 cc. of the filtrate into a 100 cc. flask, add 5 cc. of the citrate mixture and titrate with the standardised iodide solution.

*Calculation.* The grams of NaCl per cent. is  

$$\frac{(10 - \text{cc. iodide required}) \times 2}{10}.$$

## J. Phosphates.

## 414. The estimation of phosphates.

*Principle.* Urine is heated to boiling point, and titrated whilst hot with a standard solution of uranium acetate, which gives a precipitate of  $(\text{UO}_2)\text{HPO}_4$  with phosphates in acetic acid solution. Cochineal tincture is used to indicate by a change in colour when the uranium is in excess.

**Reagents required.**

1. *Acetate solution.* Dissolve 100 grams. of sodium acetate in a litre of distilled water, and add 100 cc. of strong acetic acid.

2. *Cochineal tincture*, prepared by extracting the insects with 30 per cent. alcohol and filtering after two days.

3. *Standard potassium phosphate.* Dissolve 7.672 grams. of pure recrystallised acid potassium phosphate in distilled water and make the volume up to 1 litre. 25 cc. = 0.1 gram.  $\text{P}_2\text{O}_5$ . This solution can also be prepared by measuring 28.17 cc. of the 0.2 M.  $\text{KH}_2\text{PO}_4$  described on page 24 into a 100 cc. measuring flask and making the volume up to 100 cc. with distilled water.

4. *Standard uranium acetate.* Dissolve by the aid of heat 36 grams. of pure uranium acetate in a litre of distilled water. Allow the solution to cool and then filter. Standardise the solution as follows: Into a beaker measure 25 cc. of the standard potassium phosphate, add about 25 cc. of distilled water, 5 cc. of the acetate solution and about 1 cc. of the cochineal tincture. Bring the mixture to the boiling point and titrate with the uranium acetate solution from a burette till the red tinge just changes to a green, heating the mixture to boiling just before the last few drops are added. If  $x$  cc. of the uranium solution are used, then 1 cc. of the uranium corresponds to

$$\frac{0.1}{x} \text{ gram. } \text{P}_2\text{O}_5.$$

If desired the solution can be diluted with water so that 1 cc. = 0.005 gram.

$\text{P}_2\text{O}_5$ . To effect this add  $\frac{(20 - x) \times 100}{x}$  cc. of distilled water to every 100 cc.

*Method.* In a beaker of about 100 cc. capacity place 50 cc. urine, add 5 cc. of the sodium acetate solution and about 1 cc. of the cochineal tincture. Have a burette ready containing the standardised uranium acetate solution. Heat the urine to boiling point, remove the flame and run in the uranium acetate as long as a precipitate is formed. Heat the mixture again just to boiling point, and cautiously add uranium acetate, drop by drop, till the red colour is converted to a green.

*Calculation.*

1 cc. of the uranium acetate = 0.005 gram.  $P_2O_5$ .

Thus if 50 cc. of urine require 15.2 cc. uranium, the percentage of  $P_2O_5$  is  $2 \times 15.2 \times 0.005 = 0.152$  gram.

### K. Sulphates.

Sulphates can be determined gravimetrically as barium sulphate or volumetrically by means of benzidine. The latter is much more convenient, but both methods are given below. The difficulty encountered with the gravimetric method is that of preventing adsorption of other substances. For that reason the barium must be added very slowly and the method is extremely tedious.

#### 415. The estimation of total sulphates by Folin's method.\*

Place 25 cc. of urine in a 250 cc. Erlenmeyer flask, add 20 cc. of hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water) and boil gently for 30 minutes, covering the mouth of the flask with a small watch glass. Cool the flask under the tap and dilute to about 150 cc. with water. Add 10 cc. of 5 per cent. barium chloride solution slowly, drop by drop, to the cold solution, which must not be stirred or shaken during the addition, nor for at least one hour after. Then shake well, filter through a weighed Gooch crucible (see note to Ex. 410), wash with 250 cc. of cold water, dry in an air bath, or over a very low flame. Ignite, cool and weigh.

*Calculation.* Weight of  $BaSO_4 \times 1.366 = SO_3$  per cent.

NOTES.—Instead of using a Gooch crucible a washed "Barium sulphate" filter paper may be used.

After washing and drying the ignition may be carried out in a platinum or porcelain crucible, previously weighed. After ignition, the ash should be treated with a drop of 25 per cent. sulphuric acid, cautiously dried and heated again.

A correction must be made for the weight of the ash of the paper.

\* *Journ. Biol. Chem.*, i, p. 150.

**416. The estimation of inorganic sulphates by Folin's method.**

Place 25 cc. of urine and 100 cc. of water in a 250 cc. Erlenmeyer flask. Acidify with 10 cc. of hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). Add 10 cc. of 5 per cent. barium chloride, drop by drop, as in the previous exercise, and proceed as there directed.

*Calculation.* The same as for total sulphates.

**Ethereal Sulphates.**

This can be found by difference. Total sulphates less inorganic sulphates = ethereal sulphates.

**417. The estimation of total sulphur by Benedict's method.\***

Place 10 cc. of urine in a small (7-8 cm.) porcelain or silica crucible and add 5 cc. of Benedict's sulphur reagent. Evaporate over a free flame, keeping the solution just below the boiling point, to prevent loss by spattering. When dry, raise the flame slightly until the entire residue has blackened. Raise the flame still more and heat to redness for ten minutes after the black residue (which first fuses) has become dry. Allow the dish to cool. Add 10 to 20 cc. of 1 in 4 hydrochloric acid, and heat again till the residue has completely dissolved to a clear solution. Wash the contents quantitatively into an Erlenmeyer flask, and dilute with cold water to 100 to 150 cc. Add 10 cc. of 10 per cent. barium chloride, drop by drop, and allow to stand for about an hour. Shake thoroughly and proceed as in Ex. 415.

*Calculation.* Weight of  $\text{BaSO}_4$  from 10 cc. of urine multiplied by 3.413 =  $\text{SO}_2$  per cent.

NOTE. Benedict's sulphur reagent is:

Crystallised copper nitrate, 200 grams.  
Potassium chlorate, 50 grams.  
Distilled water to 1 litre.

**Neutral Sulphur.**

This can be found by difference. Total sulphates less total sulphur = neutral sulphur.

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\* *Journ. Biol. Chem.*, vi., p. 363.

#### 418. Inorganic sulphates by the benzidine method of Rosenheim and Drummond.\*

*Principle.* The urine is acidified with hydrochloric acid and treated with an excess of benzidine hydrochloride. The sulphates are precipitated quantitatively. The precipitate is filtered off under suction, washed free from acid with water (or better with a saturated solution of benzidine sulphate) and suspended in hot water. Phenol phthalein is added, and the mixture titrated with standard soda. A pink colour does not develop until enough soda has been added to combine with the whole of the benzidine sulphate to form sodium sulphate. Benzidine sulphate, being the salt of a weak base with a strong acid, suffers hydrolytic dissociation into the base and the acid. The base is only very feebly ionised, whilst the strong acid is freely ionised, the solution in hot water thus behaving like sulphuric acid, which can be titrated with the standard soda.

##### Solutions required.

1. *Benzidine hydrochloride.* Rub up 4 grams. of pure benzidine with about 10 cc. of distilled water. Transfer with about 500 cc. of water to a 2 litre flask. Add 5 cc. of concentrated hydrochloric acid and make up to 2 litres with distilled water.

2. *Hydrochloric acid.* Dilute 1 volume of pure concentrated hydrochloric acid with 3 volumes of distilled water.

3. *Saturated benzidine sulphate.* Prepare some benzidine sulphate by adding a little sodium sulphate to 200 cc. of the benzidine hydrochloride. Collect the precipitate as described below and wash it thoroughly with cold water. Suspend it in a considerable volume of hot water and allow it to stand over-night in a cool place. Filter from the benzidine sulphate till quite clear.

4. *0.1 N. sodium hydroxide.* See appendix. The exact strength is immaterial, so long as it be accurately determined.

5. *Phenol phthalein.* A saturated solution in alcohol.

*Method.* Measure 25 cc. of the urine (filtered, if necessary) into a 250 cc. Erlenmeyer flask, with a wide neck. Add 2 cc. of the hydrochloric acid and 100 cc. of the benzidine hydrochloride. Mix and allow to stand for 10 minutes. Filter through paper and paper pulp, as described in Ex. 406. The filtrate must be crystal clear. If it is cloudy it must be passed through the filter again. Wash out the beaker with 10 cc. of the saturated benzidine sulphate and wash the precipitate with this. Repeat this at least once more. Transfer the precipitate, filter pulp and disc to the Erlenmeyer flask and wash the funnel into the flask with a jet of boiling water, using about 50 cc. of water. Any lumps of the precipitate must be broken up by use of a glass rod before the titration is commenced, or, if this is

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\* *Biochemical Journal*, viii., p. 134.

impossible, before the titration is completed. Add a few drops of the saturated solution of phenol phthalein and titrate the hot solution with the standard soda. The end point is quite sharp.

*Calculation.* 1 cc. of 0.1 N. soda  $\equiv$  4.0 mg.  $\text{SO}_3$ .

419. **Total sulphates by the benzidine method.** Measure 25 cc. of the urine into the Erlenmeyer flask, add 2 to 2.5 cc. of the hydrochloric acid and 20 cc. of distilled water. Place a funnel in the neck of the flask and boil *gently* for 20 minutes. Cool thoroughly under the tap, add 100 cc. of the benzidine hydrochloride, and proceed as directed above.

### Ethereal Sulphates.

The difference between the result of the analysis in Ex. 419 and that of Ex. 418 is the ethereal sulphate.

### Total Sulphur and Neutral Sulphur.

The urine can be oxidised by Benedict's method (Ex. 417) and the residue dissolved in hydrochloric acid. Before the benzidine method is applied the excess of free hydrochloric acid must be reduced by the addition of soda until the solution is only just acid to congo red paper. The calculation for neutral sulphur is explained in Ex. 417.



Fig. 50. Esbach's albuminometer.

### L. Albumin.

#### 420. The estimation of albumin by Esbach's method.

Fill the albuminometer to the mark U with urine. Add Esbach's reagent (Ex. 14) to the mark R. Stopper the tube, and invert it slowly several times to mix the fluids. Allow the tube to stand upright for 24 hours.

*Calculation.* The graduations on the albuminometer indicate grams. of albumin per litre.

#### 421. The estimation of albumin by Scherer's method.

Measure 50 cc. of urine into a beaker. Place it on a water bath and raise the temperature to 50° C. Add 1 per cent. acetic acid,

drop by drop, to obtain a complete separation of the protein (care must be taken to avoid an excess). Raise the temperature to boiling and keep it so for a few minutes. Filter the urine through a small paper that has previously been washed, dried and weighed. Wash the precipitate in turn with hot water, 95 per cent. alcohol and ether. Dry the paper and precipitate in an air bath at  $110^{\circ}$  C. till the weight is constant. The weight of protein in 50 cc. is obtained by subtracting the weight of the paper.

### M. Diastase.

#### 422. Wohlgemuth's method.

*Principle.* Varying amounts of urine are added to a given amount of soluble starch, and the mixture digested for 30 minutes at  $38^{\circ}$  C. After cooling, a drop of dilute iodine is added to each tube. The tubes that contain considerable amounts of urine have all the starch digested so that no colour is obtained on adding the iodine. The tube with the smallest amount of urine that completely digests the starch is found and so the diastatic value calculated (see p. 192).

#### Reagents required.

1. Stock solution of soluble starch. Accurately weigh out 2 grams. of soluble starch (see p. 395) and transfer it to a dry test-tube. Add about 10 cc. of distilled water and shake. Pour the suspension into about 70 cc. of boiling distilled water and stir well. Wash the tube three successive times with 5 cc. of distilled water, transferring the washings to the boiling solution. Now add 10 grams. of pure sodium chloride. Allow to cool and make the volume up to 100 cc. with distilled water. The solution is stable for months.
2. One per mille soluble starch in 0.5 per cent. sodium chloride. 5 cc. of the stock are diluted with distilled water to make 100 cc. This solution must be freshly prepared each day.
3. N/50 iodine, prepared from N/10 iodine (see p. 394) by diluting 2 cc. with 8 cc. of distilled water. The diluted iodine must be freshly prepared each day.

*Method.* Label a series of clean dry test-tubes 1 to 10.

Into the tubes measure the volume of urine and of distilled water stated in the table, using guarded pipettes (see Note 7).

| Tube. | c.c. of Urine. | c.c. of Water. | $d_{38^{\circ}}^{30'}$ | Tube. | c.c. of Urine diluted 1 in 10 with water. | c.c. of Water. | $d_{38^{\circ}}^{30'}$ |
|-------|----------------|----------------|------------------------|-------|---|----------------|------------------------|
| 1     | 0.5            | 0.5            | 4                      | 6     | 0.9                                       | 0.1            | 22.2                   |
| 2     | 0.4            | 0.6            | 5                      | 7     | 0.8                                       | 0.2            | 25                     |
| 3     | 0.3            | 0.7            | 6.6                    | 8     | 0.7                                       | 0.3            | 28.6                   |
| 4     | 0.2            | 0.8            | 10                     | 9     | 0.6                                       | 0.4            | 33.3                   |
| 5     | 0.1            | 0.9            | 20                     | 10    | 0.5                                       | 0.5            | 40                     |

To each tube add 2 cc. of the one per mille starch, commencing with tube 10. Mix the contents by agitation and place in a thermostat or a water bath at 38° C. for exactly 30 minutes.

Remove the tubes and place them in a beaker of cold water for 3 minutes to cool.

Arrange the tubes in order in a stand.

Commencing with tube 1 add 1 drop of the N/50 iodine to each tube and carefully note the colour produced.

Should a colour be produced and it rapidly fades, add 1 more drop of iodine to each tube.

Note the tube with the lowest number that shows a blue tinge. The next lower tube contains an amount of urine that completely digests 2 cc. of 0.1 per cent. starch in 30 minutes at 38° C.

*Calculation.* This is explained on page 192. The  $d$  values corresponding to the volumes of urine required are given in the table. Thus if tube 4 shows a bluish tint and tube 3 a red, then since 0.3 cc. of urine have digested 2 cc. of starch, then 1 cc. of urine would digest  $\frac{2}{0.3} = 6.6$  cc. starch.

So  $d = 6.6$ .

NOTES.—1. It is customary to use a freshly prepared 0.1 per cent. solution of starch in water and to make the volume of the urine up to 1 cc. with 1 per cent. sodium chloride. The author has determined that 2 per cent. starch in 10 per cent. sodium chloride is quite stable and that the results obtained agree with these found by the original method. It is suggested that the present more convenient method be adopted as a standard.

2. The  $d_{30}^{38}$  of normal urine varies between 5 and 20, with an average of 10. In acute pancreatitis and in many cases of carcinoma of the pancreas the value is high and may be over 200. In such cases the urine is still further diluted to 1 in 100 and the  $d$  calculated.

Thus if 0.006 cc. of urine is required  $d = \frac{2}{0.006} = 333$ .

It is of considerable pathological importance to note that the kidney in interstitial nephritis has difficulty in passing the diastase. The index of the urine is characteristically low in this condition.

It is high in certain cases of "toxaemia of pregnancy" (eclampsia).

3. It is important that the same amount of iodine be added to each tube. Very uneven results are obtained if varying amounts of iodine be employed.

4. Samples of the mixed 24 hours' specimen should be used.

5. The diastase in the urine is quite stable if the urine be preserved by the addition of toluol. 3 cc. are ample for an estimation.

6. The pipettes for measuring the solutions must be accurate 1 cc. pipettes graduated to 1/100 cc.

7. The end of the pipettes that are placed in the mouth should be guarded by plugs of cotton wool, to prevent contamination of the fluids with saliva. Bewildering results in class work disappeared after this precaution was rigorously enforced.



## CHAPTER XIV.

### DETECTION OF SUBSTANCES OF PHYSIOLOGICAL INTEREST.

If no indication as to the origin of the substance is available the scope of the analysis is very considerable. The following account is not intended to be exhaustive, but merely to suggest a few methods of attack. Success demands a sound knowledge of the properties and reactions of a large number of substances. Experience, practice and enterprise count for a good deal. Many substances are not detected by student analysts mainly because they forget to test for them. The hints on page 372 should be carefully studied. The student is urged to perform his tests on the smallest amount of material that is likely to give a conclusive result. With a limited supply of the substance for analysis a much greater variety of tests can thus be applied.

#### A. Fluids.

1. A portion may be neutralised and evaporated to dryness on the water bath. This allows for a subsequent extraction with strong alcohol, which serves for the separation of many substances. It should not be started until there is some indication that it may be necessary, as for the separation of sugars from proteins and polysaccharides, etc. The evaporation must be conducted in neutral solution to obviate any chemical changes produced by hot acids or alkalis.

2. Note any characteristic smell of urine, bile, etc. In such cases apply tests for characteristic constituents.

3. Note the colour and appearance of the fluid: opalescence suggests starch, glycogen, or certain protein solutions; coloured fluids suggest bile, blood or urine.

4. Note the reaction to litmus. An acid reaction excludes the presence of mucin, nucleoproteins, caseinogen, and usually earthy phosphates.

5. If acid test for free HCl by Gunsberg's test. (Ex. 246.)

6. Sprinkle some flowers of sulphur on the surface of a portion of the fluid in a test-tube. If the particles fall through the surface, bile salts are probably present. (Ex. 316.) Confirm by Pettenkofer's test. (Ex. 315.)

7. If the fluid be brown or green, apply the Huppert-Cole test (Ex. 318) for bile pigments.

8. If the fluid be red or brown, examine for blood-pigments or derivatives by Table F, page 370.

9. If there are any reasons for suspecting the presence of ferments, examine as directed on page 371. If none of the colour reactions for proteins are obtained, ferments are probably absent.

10. Examine for proteins by Millon's and the biuret reactions (Exs. 22 and 24). If they be present, proceed as directed in Table A, B or C, according to the reaction of the fluid.

11. If proteins are absent, proceed to Table E.

12. Test for uric acid if the fluid be alkaline, neutral or only faintly acid. Acidify with a drop or two of strong hydrochloric acid; uric acid may separate out as a crystalline powder. Make another portion of the solution alkaline with ammonia, saturate with  $\text{NH}_4\text{Cl}$  and apply the murexide reaction to the precipitate thus obtained. (Ex. 352.)

13. If the fluid be alkaline, treat a little with a solution of calcium chloride. A white curdy precipitate indicates the presence of soaps. (Their presence should be confirmed by the methods given in Ex. 177.)

14. Treat a portion with a little hypobromite solution. If an effervescence is obtained, test for urea by Ex. 343. If this is negative the solution may contain amino-acids or ammonium salts. The bromine test for free tryptophane (p. 217) may give a valuable indication.

Table A.

Analysis of an acid solution containing proteins.

| Add sodium carbonate solution till neutral to litmus or till the maximum precipitate has been obtained and filter.   |   |   |
|--|---|---|
| <i>Precipitate.</i> Treat with 5 c.c. of dilute NaOH on the filter paper, passing the filtrate through two or three times.   |   |   |
| <i>Residue.</i> Dissolve in nitric acid. Add ammonium molybdate & boil. Yellow ppt. indicates  | <i>Filtrate.</i> Neutralise with dilute acetic acid. Precipitate, soluble in excess of acid indicates | <i>Filtrate A.</i> Boil. Whilst boiling add 1 % acetic acid, drop by drop, to keep the reaction <i>faintly</i> acid. Filter. (If there is no heat coagulum proceed to Table D.) |
|  |   | <i>Filtrate B.</i><br>Proceed as in Table D.  |
| <i>Coagulum</i> indicates albumin or globulin. If obtained, treat a portion of Filtrate A with an equal volume of saturated ammonium sulphate solution and filter. |   |   |
| <i>Precipitate.</i> Scrape off the paper. Dissolve in a small amount of cold water and boil. Coagulum indicates  |   | <i>Filtrate.</i><br>Boil. Coagulum indicates  |
| <b>Earthy phosphates.</b>  |   | <b>Globulin.</b>  |
| <b>Metaprotein.</b>  |   | <b>Albumin.</b>   |

Table B.

Analysis of a **neutral** solution containing proteins.

|  |   |
|--|---|
| Acidify a portion with dilute acetic acid.   |   |
| <i>Precipitate.</i> (i.) <b>Bile salts with any protein.</b> Test original solution for bile salts. (Ex. 316.)<br>(ii.) <b>Mucin.</b> Insoluble in strong acetic acid.<br>(iii.) <b>Casein or nucleoprotein.</b> Soluble in strong acetic acid. Contain phosphorus. (Ex. 193.) | <i>Filtrate.</i> Neutralise and proceed as for filtrate A, Table A. |

Table C.

Analysis of an **alkaline** solution containing proteins.

|  |  |  |  |  |  |                     |  |
|--|--|--|--|--|--|---------------------|--|
| Add a drop of litmus and then acetic acid till distinctly acid. Filter.  |  |  |  |  |  |                     |  |
| <i>Precipitate.</i> (i.) <b>Bile salts with any protein.</b> Test original solution for bile salts by Hay's test. (Ex. 316.)<br>(ii.) <b>Fatty acids.</b> Test original solution for soaps by Ex. 177.<br>(iii.) <b>Mucin.</b> Insoluble in strong acetic acid.<br>(iv.) <b>Casein or nucleoprotein.</b> Soluble in strong acetic acid. Contain phosphorus. (See Ex. 193.) | <table> <tr> <td colspan="2"><i>Filtrate.</i> Neutralise with sodium carbonate.</td></tr> <tr> <td><i>Ppt.</i> Soluble in excess of alkali.</td><td><i>Filtrate.</i> Proceed as for filtrate A, Table A.</td></tr> <tr> <td colspan="2"><b>Metaprotein.</b></td></tr> </table> | <i>Filtrate.</i> Neutralise with sodium carbonate. |  | <i>Ppt.</i> Soluble in excess of alkali. | <i>Filtrate.</i> Proceed as for filtrate A, Table A. | <b>Metaprotein.</b> |  |
| <i>Filtrate.</i> Neutralise with sodium carbonate.   |  |  |  |  |  |                     |  |
| <i>Ppt.</i> Soluble in excess of alkali.   | <i>Filtrate.</i> Proceed as for filtrate A, Table A.   |  |  |  |  |                     |  |
| <b>Metaprotein.</b>  |  |  |  |  |  |                     |  |

Table D.

Examination of Filtrate B for **albumoses**, **peptones** and **gelatin**.

| Treat a portion with caustic soda and a drop of copper sulphate solution. |   |  |  |
|---|---|--|--|
| No biuret reaction.   | Positive biuret reaction. To portions of filtrate B apply Millon's and glyoxylic tests. |  |  |
|   | Negative reactions.   | Positive reactions. Saturate filtrate B with ammonium sulphate by heating with excess of solid. Cool under tap and filter.   |  |
|   | Gelatin present.  |  |  |
|   | Confirm by obtaining a precipitate by half-saturation with amm. sulphate.               | Precipitate. Mostly sticking to tube. Wash with cold saturated ammonium sulphate. Dissolve in a little boiling water and cool under tap. To portions apply biuret test (using 40 per cent. NaOH) and the glyoxylic test. If both are positive— | Filtrate. Treat 2 cc. with 4 cc. of 40 per cent. NaOH and a drop of copper sulphate. Pink colour indicates |
| Proteins absent.  |   | Albumoses.   | Peptones.  |

Table E.

Examination of a solution for **carbohydrates**.

If proteins be present they must be removed, as far as possible, by neutralising, boiling and filtering.

In any case the solution tested must be neutral.

(a) To a small portion add diluted iodine drop by drop, until an excess has been added. If a pure blue colour be obtained at any stage of the addition of iodine, starch is present. If a purple or brown colour be produced and the fluid be quite clear, erythro-dextrin is present and glycogen absent. If a blue colour be produced, or if the fluid be opalescent, proceed as follows:

To a portion of the fluid, prepared as directed above, add an equal bulk of saturated  $(\text{NH}_4)_2\text{SO}_4$ , shake vigorously, and filter through a dry paper after about ten minutes.

|   |  |  |
|---|--|--|
| <p><i>Precipitate.</i></p> <p>Scrape off the paper, dissolve in a little hot water, cool and add a drop of iodine. A blue colour shows the presence of <b>starch</b>.</p> | <p><i>Filtrate.</i> To a small portion add a drop or two of iodine. If a reddish or purple colour be produced, glycogen or dextrin is present. If the fluid be opalescent after warming, <b>glycogen</b> is present. Saturate the remainder with <math>(\text{NH}_4)_2\text{SO}_4</math> and filter.</p> |  |
|   | <p><i>Precipitate.</i><br/>Neglect.</p>  | <p><i>Filtrate.</i> Add a drop of diluted iodine, a red-brown colour shows the presence of <b>erythro-dextrin</b>.</p> |

(b) Apply Benedict's (Ex. 100) or Fehling's test (Ex. 97) for **reducing sugars**. Note that the tests do not succeed in the presence of any considerable amount of ammonium salts. Also that if albumoses, peptones or gelatin are present they should be removed by alcoholic extraction as described in Ex. 58.

(c) If a reduction be obtained, apply Barfoed's test (Ex. 101) to distinguish between mono- and di-saccharides. The osazone test (Ex. 109) also can be applied if necessary.

(d) Test for **cane-sugar** and **fructose** (see Exs. 130 and 131).

Table F.

Examine the solution spectroscopically: gradually dilute the solution, noting the spectrum at all stages of dilution.

Take the reaction of the undiluted fluid to litmus paper, washing the surplus off the paper with a stream of distilled water, if you are unable to note the reaction directly.

If the fluid be neutral or alkaline, treat it with Stokes' fluid or warm it with ammonium sulphide, and note whether the spectrum is altered by reduction. This should be done after various dilutions of the original solution.

|             |  |   |
|-------------|--|---|
| Fluid red   | Acid— <i>Acid haematoporphyrin</i> , two bands. (Ex. 307.) |   |
|             | Neutral  | Dilute till two bands are well seen and then reduce.  |
|             |  | <i>Oxyhaemoglobin</i> , the two bands merge into one faint band (Ex. 293.)<br><i>CO-haemoglobin</i> , the two bands are unaltered. (Ex. 296.) |
|             | Alkaline   | <i>Alkaline haematoporphyrin</i> , four bands, converted into acid haematoporphyrin by strong acids. (Exs. 307 and 308.)                      |
|             |  | <i>Haemochromogen</i> , two bands in green, one much more distinct than the other, unaffected by reducing reagents. (Ex. 305.)                |
| Fluid brown | Acid— <i>Acid haematin</i> , band in red. Ex. 301.)        |   |
|             | Neutral.   | <i>Methaemoglobin</i> , band in red: gives spectrum of oxyhaemoglobin and then of reduced haemoglobin if reduced. (Ex. 299.)                  |
|             | Alkaline   | <i>Alkaline haematoporphyrin</i> —four bands. (Ex. 308.)  |
|             |  | <i>Alkaline haematin</i> , faint band in red, converted to haemochromogen by reducing reagents. (Exs. 303, 305.)                              |

### Enzymes.

In testing for enzymes it is important to note the reaction. It is not necessary to determine the exact  $P_H$ , but trials should be made with litmus, followed by phenol-red and phenol-phthalein for alkaline solutions, and methyl-red and brom-phenol-blue (or thymol-blue) for acid solutions. In this way certain valuable indications may be obtained.

The next point to remember is that all tests must be made in parallel with a control. In the control test, the solution is well boiled to destroy any enzyme that may be present: in other respects it is carried out exactly as the test proper. Without this precaution it is quite impossible to make any safe deduction.

To test for proteolytic enzymes see if the solution will clot calcified milk (Exs. 252 and 257). The solution should be nearly neutralised before applying the test, but if it is acid it must not be made alkaline (see Ex. 253). If this test is positive, pepsin can be identified by the method given in Ex. 248. Rennin can be distinguished from pepsin by Ex. 256, though it takes a good deal of time. It is unusual to find a rennin solution free from pepsin, but many commercial pepsins are practically free from true rennin. Trypsin can be identified by Ex. 258. If the solution be made faintly alkaline to phenol-phthalein, incubated for 10 mins. at  $38^{\circ}\text{C}$ ., and then neutralised, to litmus, pepsin and rennin are destroyed. If the solution now clots milk, trypsin is present.

Diastatic enzymes are probably absent if the solution is strongly acid or alkaline. The reaction and salt content are factors of importance. The best method of testing for these enzymes is some modification of Ex. 237, adding the buffer and the salt for the reasons given in that exercise. The solution should be carefully neutralised to litmus before making the tests.

The enzymes acting on the disaccharides are usually more difficult to identify. Sucrase is sometimes found in a very active condition, but the tests for maltase and lactase generally require an incubation period of at least 15 hours. For details see Exs. 266-268.

Lipase is rather unstable to acids. For tests see Ex. 168.

### A few special hints on the examination of physiological fluids.

1. It is impossible to obtain a heat coagulum of albumin or globulin in an acid or alkaline fluid. The reaction must be *neutral* or only very faintly acid.

2. A little litmus solution in the fluid does no harm, and often reminds one that the reaction changes after boiling (owing to the evolution of  $\text{CO}_2$ ).

3. In testing for peptones, after removing the albumoses by saturation with ammonium sulphate, the biuret test succeeds only if at least two volumes of 40 per cent. soda are used. The test will not be obtained with the ordinary 5 per cent. soda (see notes to Ex. 57).

4. Gelatin reacts very much like the albumoses, except that it does not yield the glyoxylic reaction. It can be precipitated by half-saturation with ammonium sulphate. If the precipitate is collected, squeezed and dissolved in a *very little* hot water, the solution will often set after being thoroughly cooled for some time.

5. It is impossible to obtain Fehling's or Benedict's test for the reducing sugars in the presence of any considerable amount of ammonia or ammonium salts.

6. The sugars reduce only in an alkaline medium. If the fluid under examination be acid, it must be neutralised before boiling with the Fehling's or Benedict's solution.

7. In testing for cane sugar do not forget that starch and the dextrins are hydrolysed to glucose by boiling acids. But whereas



cane sugar is hydrolysed very easily, starch, etc., are only slowly acted on.

8. Starch, glycogen and the erythro-dextrins do not give any colour with iodine solutions, if the reaction of the fluid be alkaline. If this be the case, make the reaction acid with acetic acid.

9. The proteins interfere with the iodine tests for these substances, and should therefore as far as possible be removed before testing for the polysaccharides.

10. Fat is insoluble in water, so do not waste time in testing an ordinary solution for fats.

11. The only reliable test for urea is the urease test (Ex. 343). In this connection it must be remembered that urea is soluble in alcohol, and can thus be separated from the proteins and other substances likely to interfere owing to their "buffer" action.

12. Ammonium chloride is a very valuable reagent in testing for uric acid or urates. The only other physiological substance precipitated by it is soap.

13. Never omit "control" tests when investigating the ferment action of a solution.

14. Use "carmin fibrin" in testing for pepsin; never when testing for trypsin.

15. In testing solutions for pigments, examine spectroscopically in various dilutions. Note the reaction of the fluid; it is no good looking for haemochromogen in a markedly acid solution.

16. Creatinine, acetone, aceto-acetic acid and lactic acid can usually be identified by specific colour reaction, though the latter generally involves an extraction with ether. Creatine can only be identified after conversion to creatinine, and then an estimation of total creatinine is necessary.

17. A solution of amino-acids evolves nitrogen gas with nitrous acid and also with alkaline hypobromites. Ammonia can be removed by gentle boiling in an open dish with a little alkali.

### B. Solids.

1. Examine a little microscopically, both dry and with the addition of a drop of water. Look for starch grains, crystals of urea, uric acid, urates, leucine, tyrosine, cholesterol, and haemin scales.

2. Heat a small amount of the solid in a dry tube, at first gently and then more strongly.

(a) If sublimation take place and an odour of amylamine be given off, leucine is present.

(b) If sublimation take place and a strong smell of ammonia be evolved, urea is indicated.

(c) A smell of phenol and nitro-benzol indicates tyrosine.

(d) A smell of burning feathers indicates proteins, gelatin, etc.

(e) A smell of acrolein indicates fats.

3. Boil some of the solid with a small amount of water in a tube, cool under the tap and leave the test-tube in a beaker of cold water for 10 minutes. If gelatin be present, the solution will set to a jelly. (Starch, if present, may form a thick paste, which may be confused with the clean jelly given by gelatin. If the tube be subsequently placed in boiling water, gelatin becomes quite limpid, whilst starch remains thick.)

4. If the solid be of a dark brown or red colour, boil a portion with dilute alkali, filter, heat the filtrate with Stokes' fluid or ammonium sulphide, and examine for the spectrum of haemochromogen. (Ex. 305.) If this be obtained, the solid contains dried blood or haematin. Confirm by obtaining haemin crystals. (Ex. 309.)

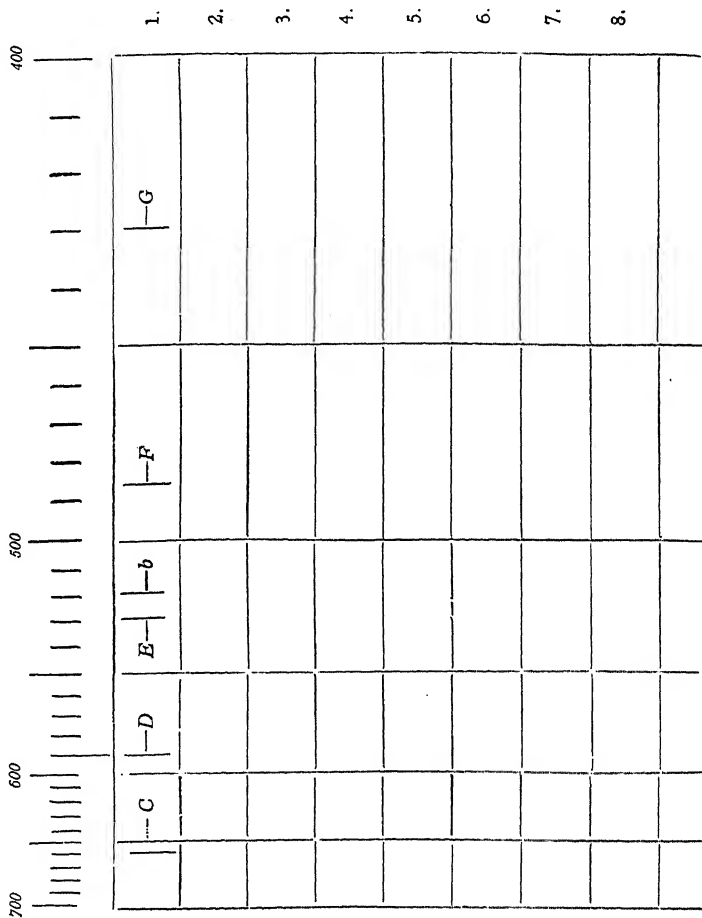
5. The table on the next page can be followed, but the method adopted will depend on the indications obtained by preliminary tests. It is advisable to test for starch before deciding on a plan of operation.

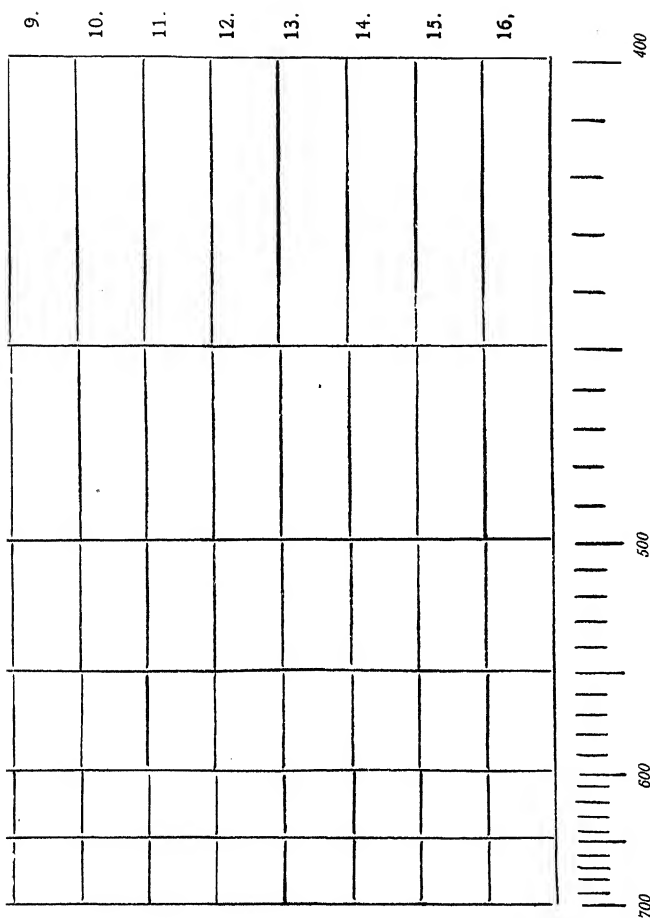
### Analysis of a Solid for substances of Physiological Interest.

Heat a considerable amount of the solid with strong alcohol on a water bath, stirring well for some time. Remove the dish from the bath, and filter the alcohol into a dry vessel. Extract the residue once more with alcohol, filter and mix the alcohols.

|   |   |  |   |
|---|---|--|---|
| <i>Alcoholic Solution</i> Evaporate to dryness on the water bath. Add 5 c c. of water, heat and stir. | <i>Residue insoluble in alcohol.</i> Treat with water, warm to about 40° C., and add acetic acid till the reaction is just acid. Cool and filter. |  |   |
| <i>Residue.</i> May contain fats, fatty acids or cholesterolin.                                       | <i>Aqueous Solution.</i> May contain urea, reducing sugars, cane sugar, bile-salts, soaps.  | <i>Solution.</i> Test for albumoses and peptones (Table D). Glycogen and dextrin. Earthy phosphates. | <i>Residue.</i> Treat with 2 per cent. Na <sub>2</sub> CO <sub>3</sub> , shake well and filter. |
|   |   | <i>Solution.</i> Test for urates (by Schiff's test) (Ex. 353), nucleo - proteins and casein.         | <i>Solution.</i> Test for starch and urates.  |
|   |   |  | <i>Residue.</i> Test for coagulated proteins.   |

# CHART FOR RECORDING THE ABSORPTION-SPECTRA OF PIGMENT SOLUTIONS.





1. Fraunhofer's lines
2. Oxy-haemoglobin (dilute)
3. Oxy-haemoglobin (medium)
4. Oxy-haemoglobin (strong)
5. Reduced haemoglobin
6. CO-haemoglobin
7. Methaemoglobin (strong)
8. Methaemoglobin (dilute)
9. Acid haematin
10. Acid haematin in ether
11. Alkaline haematin
12. Haemochromogen
13. Haematoporphyrin (Acid)
14. Haematoporphyrin (Alkaline)
15. Urobilin
16. Pettenkofer's reaction

| NAME.....  | SEX.....        | AGE.....        | WEIGHT.....           |
|--|-----------------|-----------------|-----------------------|
| Volume of 24 hours' urine                                      |                 |                 |                       |
|  | Grams per cent. | Grams per diem. | Oz.                   |
| Urea   |                 |                 | Per cent. of total N. |
| Ammonia  |                 |                 |                       |
| Uric Acid  |                 |                 |                       |
| Creatinine   |                 |                 |                       |
| Creatine   |                 |                 |                       |
| Undetermined N.  |                 |                 |                       |
| TOTAL NITROGEN   |                 |                 |                       |
| Ammonia plus Amino-acid Nitrogen                               |                 |                 |                       |
| Amino-acid Nitrogen  |                 |                 |                       |
| Total Sulphates, as SO <sub>3</sub>                            |                 |                 |                       |
| Inorganic SO <sub>3</sub>                                      |                 |                 |                       |
| Ethereal SO <sub>3</sub>                                       |                 |                 |                       |
| Neutral SO <sub>3</sub>  |                 |                 |                       |
| Acetone plus Aceto-acetic Acid                                 |                 |                 |                       |
| β-oxy-butyric Acid (as acetone)                                |                 |                 |                       |
| Sodium chloride  |                 |                 |                       |
| P <sub>2</sub> O <sub>5</sub>                                  |                 |                 |                       |
| Date when passed.....  |                 |                 |                       |
| Titratable Acid to P <sub>H</sub> = 7.45 in cc. of 0.1 N. Acid |                 |                 |                       |
| Ammonia in cc. of 0.1 N. Acid                                  |                 |                 |                       |
| Titratable Acid Ratio =  |                 |                 |                       |
| Ammonia P <sub>H</sub> =                                       |                 |                 |                       |
| Δ =  |                 |                 |                       |
| Δ =  |                 |                 |                       |
| % NaCl =   |                 |                 |                       |
| Diastatic Index =  |                 |                 |                       |
| Amount or Nature.  |                 |                 |                       |
| Sugar  |                 |                 |                       |
| Albumin  |                 |                 |                       |
| Urobilin   |                 |                 |                       |
| Bile pigments  |                 |                 |                       |
| Bile salts   |                 |                 |                       |
| Indican  |                 |                 |                       |
| Blood  |                 |                 |                       |
| Deposit  |                 |                 |                       |
| Bacteria   |                 |                 |                       |
| Pus  |                 |                 |                       |
| Remarks by Physician   |                 |                 |                       |
| Remarks by Analyst   |                 |                 |                       |

C

## APPENDIX.

## WEIGHTS AND MEASURES.

- 1 grain = .0648 gram.
- 1 ounce = 437.5 grains = 28.3595 grams.
- 1 lb. = 16 oz. = 7000 grains = 453.5925 grams.
- 1 gram. = 15.432 grains.
- 1 kilogram = 1000 grams = 2.2046 lbs.
- 1 minim = .059 cc.
- 1 fluid drachm = 60 minims = 3.55 cc.
- 1 fluid ounce = 8 fluid drachms = 28.4 cc.
- 1 pint = 20 fluid oz. = 567.9 cc.
- 1 cc. = 16.9 minims.
- 1 litre = 1000 cc. = 35.2 fluid oz. = 1.76 pints.
- 1 gallon = 8 pints = 4.542 litres.
- 1 gallon distilled water = 10 lbs.
- 1 inch = 2.54 cm.
- 1 inch = 2.54 cm.
- 1 foot = 30.48 cm.
- 1 yard = 91.44 cm.
- 1 cm. = .39 in.
- 1 metre = 39.37 in.
- 1 litre of hydrogen at 0° C. and 760 mm. Hg. = 0.0896 grams.

TENSION OF AQUEOUS VAPOUR  
in millimetres of mercury from 8° to 20° C.

| °C.  | mm.  | °C.  | mm.  | °C.  | mm.  |
|------|------|------|------|------|------|
| 8    | 8.0  | 14   | 11.9 | 20   | 17.4 |
| 8.5  | 8.3  | 14.5 | 12.3 | 20.5 | 17.9 |
| 9    | 8.6  | 15   | 12.7 | 21   | 18.5 |
| 9.5  | 8.9  | 15.5 | 13.1 | 21.5 | 19.1 |
| 10   | 9.2  | 16   | 13.5 | 22   | 19.6 |
| 10.5 | 9.5  | 16.5 | 14   | 22.5 | 20.2 |
| 11   | 9.8  | 17   | 14.4 | 23   | 20.9 |
| 11.5 | 10.1 | 17.5 | 14.9 | 23.5 | 21.5 |
| 12   | 10.5 | 18   | 15.3 | 24   | 22.2 |
| 12.5 | 10.8 | 18.5 | 15.8 | 24.5 | 22.9 |
| 13   | 11.2 | 19   | 16.3 | 25   | 23.5 |
| 13.5 | 11.5 | 19.5 | 16.8 |      |      |

INTERNATIONAL ATOMIC WEIGHTS.

| Revised<br>o = 16 |     |        | Revised<br>o = 16 |     |        |
|-------------------|-----|--------|-------------------|-----|--------|
| Barium            | Ba. | 137.37 | Mercury           | Hg. | 200.6  |
| Bromine           | Br. | 79.92  | Nitrogen          | N.  | 14.01  |
| Calcium           | Ca. | 40.07  | Oxygen            | O.  | 16     |
| Carbon            | C.  | 12.005 | Phosphorus        | P.  | 31.04  |
| Chlorine          | Cl. | 35.46  | Potassium         | K.  | 39.10  |
| Copper            | Cu. | 63.59  | Silver            | Ag. | 107.88 |
| Hydrogen          | H.  | 1.008  | Sodium            | Na. | 23.    |
| Iodine            | I.  | 126.92 | Sulphur           | S.  | 32.06  |
| Iron              | Fe. | 55.84  | Tungsten          | W.  | 184.   |
| Lead              | Pb. | 207.2  | Uranium           | U.  | 238.2  |
| Magnesium         | Mg. | 24.32  | Zinc              | Zn. | 65.37  |
| Manganese         | Mn. | 54.93  |                   |     |        |



## SPECIFIC GRAVITIES TABLES.

## I. SULPHURIC ACID.

| Sp. Gr.<br>$\frac{15^{\circ}}{4^{\circ}}$ | Gms. of<br>$H_2SO_4$<br>in 100 cc. | Sp. Gr.<br>$\frac{15^{\circ}}{4^{\circ}}$ | Gms. of<br>$H_2SO_4$<br>in 100 cc. |
|---|------------------------------------|---|------------------------------------|
| 1.840                                     | 175.9                              | 1.552                                     | 100                                |
| 1.838                                     | 173.9                              | 1.542                                     | 98.1                               |
| 1.835                                     | 171.7                              | 1.520                                     | 93.6                               |
| 1.833                                     | 170.4                              | 1.492                                     | 88.95                              |
| 1.830                                     | 168.5                              | 1.420                                     | 74                                 |
| 1.825                                     | 166.1                              | 1.380                                     | 66.2                               |
| 1.815                                     | 161.8                              | 1.295                                     | 50                                 |
| 1.800                                     | 156.4                              | 1.200                                     | 32.8                               |

## II. HYDROCHLORIC ACID.

| Sp. Gr.<br>$\frac{15^{\circ}}{4^{\circ}}$ | Gms. of<br>HCl<br>in 100 cc. | Sp. Gr.<br>$\frac{15^{\circ}}{4^{\circ}}$ | Gms. of<br>HCl<br>in 100 cc. |
|---|------------------------------|---|------------------------------|
| 1.160                                     | 36.6                         | 1.133                                     | 30                           |
| 1.155                                     | 35.3                         | 1.113                                     | 25                           |
| 1.152                                     | 34.5                         | 1.091                                     | 20                           |
| 1.150                                     | 34.0                         | 1.056                                     | 12                           |
| 1.145                                     | 32.8                         | 1.047                                     | 10                           |
| 1.140                                     | 31.5                         | 1.0375                                    | 8                            |

## APPENDIX.

## III. SODIUM AND POTASSIUM HYDROXIDES.

| Sp. Gr.<br>$\frac{15^{\circ}}{4^{\circ}}$ | Gms. of<br>NaOH<br>in 100 cc. | Gms. of<br>KOH<br>in 100 cc. |
|---|-------------------------------|------------------------------|
| 1.634                                     | —                             | 94                           |
| 1.615                                     | —                             | 90.2                         |
| 1.530                                     | —                             | 75.6                         |
| 1.438                                     | 57.5                          | —                            |
| 1.397                                     | 50.6                          | 54.3                         |
| 1.370                                     | 46.2                          | 50.6                         |
| 1.332                                     | 40.0                          | 45.1                         |
| 1.190                                     | 20                            | 25.5                         |

## IV. AMMONIA.

| Sp. Gr.<br>$\frac{15^{\circ}}{4^{\circ}}$ | Gms.<br>NH <sub>3</sub><br>in 100 cc. | Sp. Gr.<br>$\frac{15^{\circ}}{4^{\circ}}$ | NH <sub>3</sub><br>in 100 cc. |
|---|---------------------------------------|---|-------------------------------|
| .880                                      | 31                                    | .896                                      | 26.6                          |
| .882                                      | 30.83                                 | .898                                      | 26.05                         |
| .884                                      | 30.14                                 | .900                                      | 25.5                          |
| .886                                      | 29.46                                 | .902                                      | 24.94                         |
| .888                                      | 28.86                                 | .906                                      | 23.83                         |
| .890                                      | 28.26                                 | .910                                      | 22.74                         |
| .892                                      | 27.70                                 | .920                                      | 20.01                         |
| .894                                      | 27.15                                 | .926                                      | 18.42                         |

## V. ALCOHOL.

| Sp. Gr.<br>15.56° | Volume<br>per cent. | Sp. Gr.<br>15.56° | Volume<br>per cent. |
|-------------------|---------------------|-------------------|---------------------|
| ·79391            | 100                 | ·83065            | 91                  |
| ·79891            | 99                  | ·83400            | 90                  |
| ·80359            | 98                  | ·86395            | 80                  |
| ·80800            | 97                  | ·87740            | 75                  |
| ·81217            | 96                  | ·89010            | 70                  |
| ·81616            | 95                  | ·90214            | 65                  |
| ·81997            | 94                  | ·91358            | 60                  |
| ·82365            | 93                  | ·92439            | 55                  |
| ·82721            | 92                  | ·93445            | 50                  |

## BOILING POINTS.

|                    |     |     |     |       |
|--------------------|-----|-----|-----|-------|
| Acetic acid        | ... | ... | ... | 119   |
| Acetone            | ... | ... | ... | 56.5  |
| Alcohol, anyl      | ... | ... | ... | 129.6 |
| „ butyl, normal    | ... | ... | ... | 117   |
| „ butyl, iso       | ... | ... | ... | 106   |
| „ butyl, secondary | ... | ... | ... | 99.8  |
| „ caprylic         | ... | ... | ... | 179.5 |
| „ ethyl            | ... | ... | ... | 78.3  |
| „ methyl           | ... | ... | ... | 66    |
| Benzene            | ... | ... | ... | 80    |
| Carbon bisulphide  | ... | ... | ... | 46.2  |
| Chloroform         | ... | ... | ... | 63    |
| Ether...           | ... | ... | ... | 34.18 |
| Toluol             | ... | ... | ... | 111   |

|                               | Sp. Gr. | Gms. in 100 cc. |
|-------------------------------|---------|-----------------|
| Nitric acid .. .. .           | 1.42    | 99              |
| Acetic acid, "glacial" .. ..  | 1.06    | 111.1           |
| Acetic acid, "strong" .. ..   | 1.044   | 33              |
| Sodium carbonate, "saturated" | 1.15    | 16.2            |

### STANDARD ACIDS AND ALKALIES.

A *normal* solution of a substance contains in 1000 cc. that weight in grams, which corresponds to 1 equivalent in grams, of available hydrogen (1.008 grams,) or its equivalent.

Thus normal hydrochloric acid contains  $35.46 + 1.008 = 36.468$  grams. of HCl per litre.

Normal sulphuric acid contains

$$\frac{2 \cdot 016 + 32 \cdot 07 + 64}{2} = 49.043 \text{ grams. of } \text{H}_2\text{SO}_4 \text{ per litre.}$$

It is customary to employ normal, half-normal, fifth-normal, etc., solutions according to circumstances. But it is often much more convenient to determine the exact strength of a solution than to adjust it to some even fraction. For this reason it is better to express the normality as a decimal coefficient rather than as a fraction. Thus, suppose an acid be found by titration against a known standard to be 0.107 N, it can be labelled as such and used when a solution about one-tenth normal is convenient, the necessary adjustment in the calculation being very simple. The relationship is not so obvious if it be labelled  $\frac{N}{9.346}$ .

In the author's experience the simplest and most reliable starting point for the preparation of standard acids and alkalies is  $\text{CO}_2$  - free, sodium hydroxide, made and stored as described on p. 26. From such a stock it is a simple matter to prepare acids or alkalies of any desired concentration. For further details concerning the preparation and storage of the alkali see p. 322.

Thus, suppose that 0.1 N.HCl be required. Dilute pure concentrated hydrochloric acid about 90 times with distilled water, measure out 25 cc. and titrate it with the standard alkali, using either methyl red or phenol phthalein as the indicator. Suppose that the 25 cc. of dilute acid require 13.8 cc. of alkali which has been found to be 0.1965 N. Then the normality of the acid is  $\frac{13.8}{25} \times 0.1965 = 0.1085$  N.

The acid can be used as such, or if exactly 0.1 N. be required, then 8.5 cc. of distilled water is added to every 100 cc. of the acid, thus bringing it to the desired concentration.

It is important to note that acids and alkalies act on glass, and thereby suffer a change in concentration. This is practically avoided by storing in bottles that have been coated internally with a fairly thick layer of paraffin wax.

## PIPETTES, ETC.

*Delivery.* In using an ordinary single-volume pipette the fluid is drawn by suction just above the mark and closed with the finger. The lower end of the pipette is then allowed to touch the side wall of the bottle or beaker and the fluid run out till the meniscus is exactly at the mark, the eye being level with the meniscus. The fluid is then allowed to run out into the desired vessel and is then drained for 15 seconds with its point touching the wall of the vessel. The majority of pipettes are calibrated for such a delivery, but for certain operations the author prefers to use pipettes which are calibrated in such a way that they have to be blown out after draining as above for 15 secs. The reason why these are sometimes preferable is that the amount left in the nozzle of the pipette after drainage may alter considerably with variations in the surface tension of the fluid measured. It is suggested that such pipettes calibrated for delivery by blowing should be engraved with the letter "B" to distinguish them from pipettes calibrated for drainage "D."



Fig. 51.  
Ostwald  
pipette.

*Ostwald pipettes* (see fig. 51) are always calibrated for delivery by being completely blown out. The orifice must be so narrow that it takes about 30 seconds for the delivery of 1 cc. They are filled by suction to above the mark and then closed with the finger. The exterior is wiped with a piece of filter paper and the fluid run to the mark by holding the point on the filter paper. The fluid is then allowed to fall out by its own weight, the delivery being completed by blowing out whilst drawing the point of the pipette up the sides of the receiving vessel.

*Burettes.* The chief precautions to be taken are to allow time for proper drainage, and to be sure that the meniscus is read in the same way at every operation. The author prefers to use burettes that have the marks engraved as complete circles, and to read the meniscus by means of a piece of black paper held behind the burette. The black paper is pasted on to a piece of white card, which is sharply folded at the black edge (see fig. 52). The black edge is held against the back of the burette a trifle below the meniscus, the slanting white card reflecting the light. The meniscus appears as a very sharp black line. In order to avoid parallax it is important that the eye should be exactly at the level of the meniscus. To ensure this for very accurate work the author has constructed the device shown in fig. 53. Should the fluid be run rapidly out of a burette ample time must be allowed for proper drainage, the meniscus gradually moving upwards as the fluid runs down the side of the burette. Details of the methods employed for the calibration of pipettes and burettes will be found in most standard works on Quantitative Chemical Analysis.\* It is essential that pipettes and burettes should be clean and free from grease. Very considerable errors can be caused by variations in

\* *Representative Procedures in Quantitative Chemical Analysis*, by F. A. Gooch (Chapman & Hall, London, 1916), can be recommended.

the amount of fluid adhering in the form of drops in a greasy pipette or burette. Should the burette have a glass stopcock this must be greased. Under these circumstances the fluid must always be run out of the burette by the stopcock. If it is emptied by opening the tap and inverting, the inner wall of the vessel is almost certain to become greasy. When this occurs,

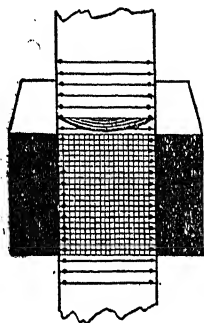


Fig. 52.

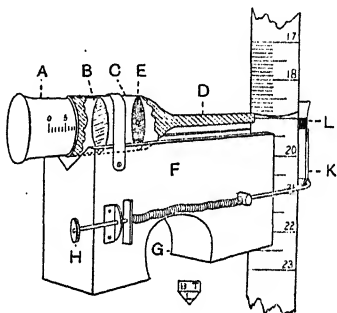


Fig. 53.

#### Author's device for reading burettes.

A is a draw tube containing a lens B. E is a paper disc pierced with a small hole. The tube C and D are blackened. The whole is fastened to a wooden block, F. This is firmly held to the burette by a clip and spring. By placing the finger in the groove G and pressing with the thumb on H, the tube can be moved up and down until the meniscus is sighted. L is a piece of paper, the lower half of which is blackened. The device is for reading to one-tenth of the ordinary graduations of the burette. The nearest tenth is best obtained by the method described above.

wash the burette out with water. Fill it with strong (40%) soda. Run this out and then wash it out repeatedly with tap water. Now fill the burette with chromic acid cleaning fluid and allow it to stand over-night. Wash out as before. The burette will now keep free from grease for some time if properly used.

## FOLIN'S FUME-ABSORBER.

Since the fumes arising from the incineration of a substance with boiling sulphuric acid are extremely irritating, that operation should be conducted in a fume chamber or under a hood. But it is preferable to use the very convenient apparatus devised by Folin, since the removal of the condensation water materially accelerates the incineration.

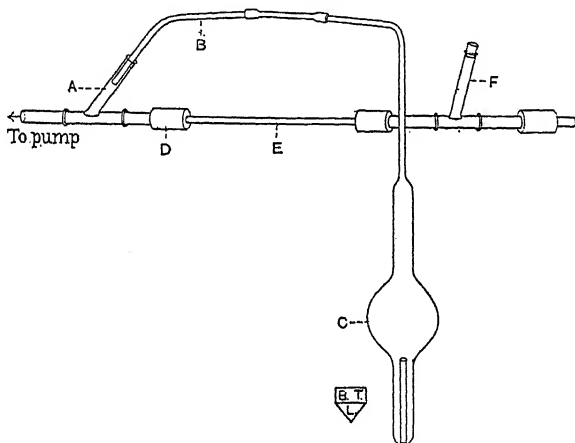


Fig. 54. Folin's fume-absorber.

The apparatus consists of a bulb C ( $1\frac{1}{2}$  inches in diameter) blown into a piece of  $\frac{3}{8}$ ths. tubing. The lower end has blown into it an open piece of narrow tubing  $2\frac{1}{2}$  inches in length. The bulb rests on the neck of the flask or test-tube in which the incineration is conducted. To the upper end of the tube is fixed a piece of narrow glass tubing which is bent at a convenient angle and connected by a short length of rubber tubing to a glass tube B. This is of such a size that it just slips into one limb (A) of a T-piece. This is fastened to a board or shelf by metal clips. One end of the horizontal limb of the T-piece is connected to a suction pump, the other end being joined by a piece of pressure tubing (D) and a length of metal tubing (E) to another T-piece. This can be connected to another fume-absorber. One good pump is sufficient for 3 absorbers. Those not in use should be stoppered with corks. It is sometimes necessary to fit a rubber collar on to B, so that good suction is obtained through C. Owing to the rubber joints the angles of the limbs A and F can be varied to suit the heights of the vessels in which the incineration is being conducted.

The fumes are carried over by the air current into the pump, a wash bottle containing soda being interposed to prevent damage. The condensation water collects in the pocket below C and can be removed by inverting the fume-absorber at the end of the operation.

By inverting a funnel over an evaporating basin, and arranging the apparatus so that the end of the funnel fits loosely into the neck of the absorber, the fumes from boiling nitric acid can be carried off.

## COLORIMETERS.

A high grade colorimeter is a necessary adjunct of a Biochemical Laboratory, a number of important analyses being made by its use.

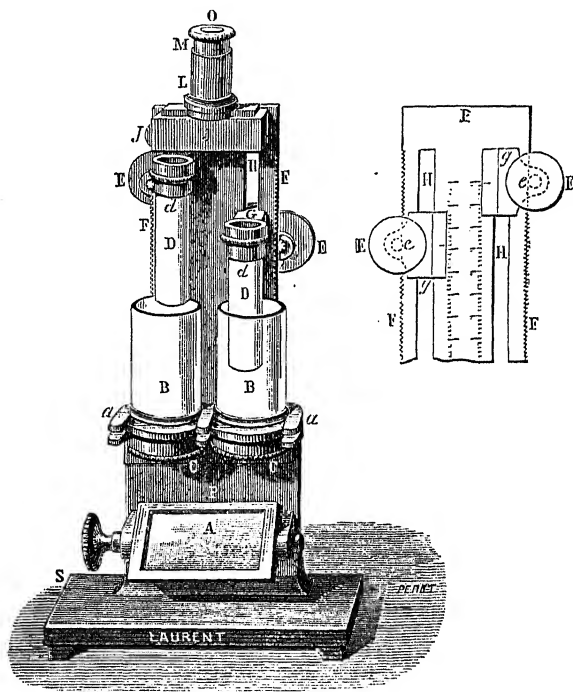


Fig. 55. Duboscq's Colorimeter.

Inset shows construction of vernier scale.

The best known instrument is that of Duboscq, which is shown in fig. 52. The standard solution is placed in one of the cups, B, and the unknown solution in the other cup. The plungers, D, are either cylindrical hollow cups, closed at the bottom, or, preferably, are made of a solid piece of optically clear glass. They can be moved up and down by turning the screw E, which works on a rack and pinion. The height of the bottom of the plungers from the bottom of the cups, that is the depth of the solution used, can be read by means of a scale and vernier at the back of the instrument. The standard is set at a given height (say 15 mm.) and the height of the other plunger adjusted until exact equality of tint is obtained. The light is reflected through the solutions from A, which is either a mirror or a piece of opal glass. After passing through the layers of the fluids on the two sides the light falls on to the prisms shown in K of fig. 56. These prisms are contained in the case marked J in fig. 55. The light then passes through the eye piece as shown.



There are several important points of detail that must be attended to before accurate results can be obtained.

(1) See that the zero points of the scales are correct, by carefully lowering the plungers until they touch the cups and noting the readings.

(2) See that the prisms and eye piece are clean. Specks of dust seen in the field are apt to lead to erroneous judgments. The prisms can be removed and carefully cleaned with a pointed match covered with two or three layers of silk. Great care must be taken to avoid breaking the prisms away from the cement.

(3) See that the illumination of the two fields is equal. This is best tested by placing a coloured solution (such as a 2.5 per cent. solution of potassium dichromate) in both cups, placing one plunger at 15 mm. and adjusting the other until the two fields have an identical appearance. The other plunger should also be at 15 mm. Attention must be paid to the point considered below.

(4) Folin has suggested that the best place for an instrument is in the middle of the Laboratory, so that the eye is not dazzled by the light from the window. Retinal fatigue will undoubtedly cause very serious errors and inconsistencies, and Folin's recommendation is valuable.

(5) A comfortable body position is important. For some reason the best results are obtained when the observer is in an unstrained position. Folin suggests that the best way of using the apparatus is to place it on a stool about the same height as an ordinary chair, and to sit at the side of the instrument. His method of reading the unknown is to place about the standard solution into both cups and to set them at the same height. The instrument being adjusted, both fields should look alike, and the eye gets accustomed to the appearance. The standard in one cup is replaced by the unknown, and one very careful observation is taken. When making a series of comparisons, he re-reads the standard against itself after each of two unknowns.

Kober's Colorimeter\* is a great advance on Duboscq's. The manufacturers (Klett Manufacturing Co., New York, U.S.A.) kindly sent one to Cambridge for trial. It has been found admirable in every way, and is always used now in preference to the various patterns of Duboscq's. Kober's instrument can also be used as a Nephelometer, *i.e.* for estimating substances

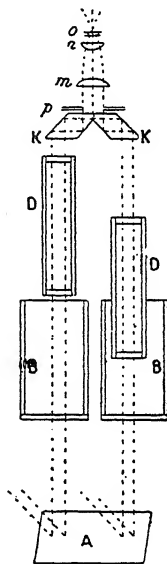


Fig. 56. Diagram of path of rays in Duboscq's Colorimeter. Below are representations of the appearance of the field under different conditions, that on the left with no fluid in B, and that on the right when the tints are matched.

\* *Journ. of Biol. Chem.*, xxix., p. 155.

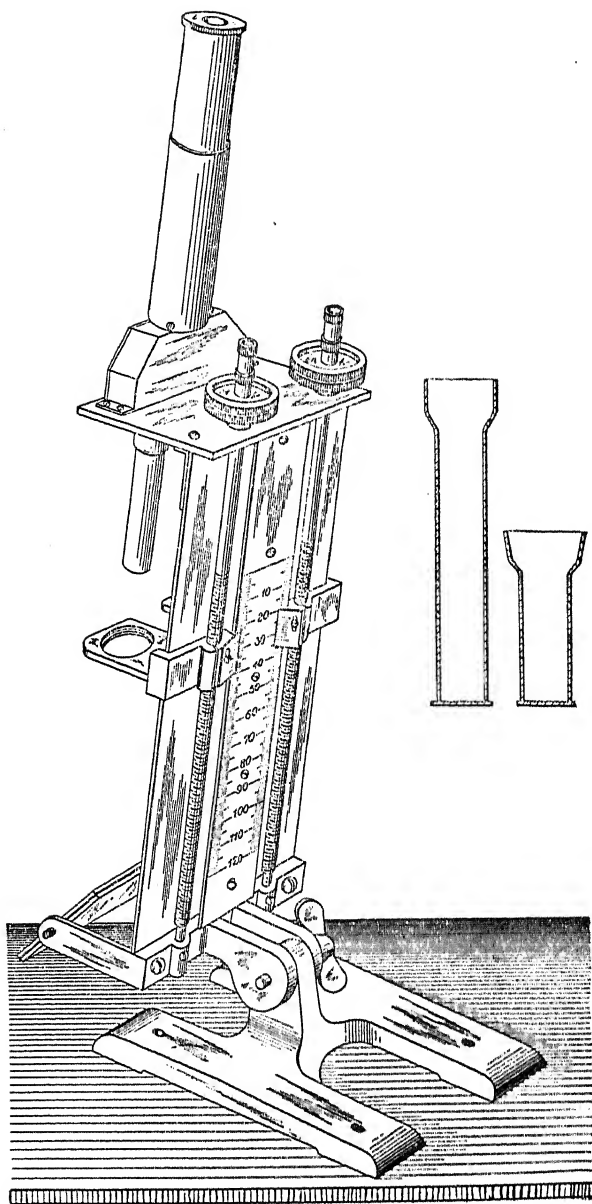


Fig. 57. Kober's Colorimeter. Inset shews the form of two of the cups.

by the density of a cloudy precipitate. Though this book does not contain an example of this method of analysis, it is of growing importance, being valuable when only very small amounts of material are available. It is also supplied, if desired, with an excellent lamp house, so that it can be used at night. In fact, it is more satisfactory to use artificial light, since by means of mirrors the illumination of the two fields can be made exactly equal.

The instrument is shewn in fig. 57, and it will be seen that the plungers are fixed and the cups movable. This does away with the space between the top of the cup and the prism, which is apt to allow an indefinite amount of light through in a Duboscq. The prisms are enclosed and so remain free from dust. The use of dark glass for the sides of the cups and the plungers, the absence of cements, improved mechanical arrangements for adjusting the zero and moving the cups, and the reduction in the volume of fluid necessary, all combine to make the instrument nearer perfection than anything yet introduced.\*

The calculations necessary in colorimetric work are very simple. The assumption is that the depth of colour is proportional to the concentrations of the substance in the standard and in the unknown. Also that the depth of field of the solutions that must be taken to get equality of tint vary inversely as the intensity of the colour produced, and therefore as the concentrations in the standard and unknown. So if the standard contains a certain amount of material in a given volume and the unknown is made up to the same volume, then

$$\frac{\text{Amount in standard}}{\text{Amount in unknown}} = \frac{\text{Reading of unknown}}{\text{Reading of standard}}$$

---

\* Both instruments can be obtained from Messrs. Baird and Tatlock (London).

## TORSION BALANCE.

This instrument is of value for the rapid weighing of small amounts of substances, such as blood taken from a finger-prick, etc.

The instrument is used as follows for the weighing of blood on a piece of absorbing paper.

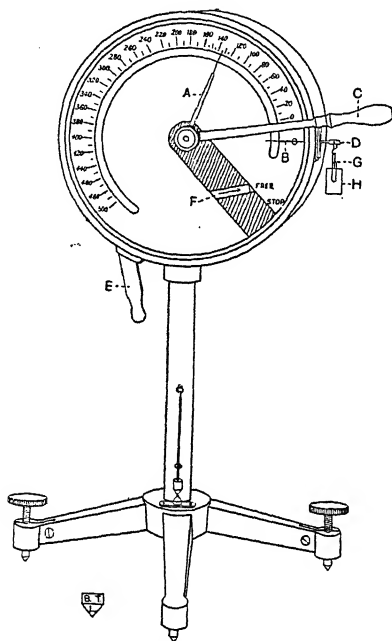


Fig. 58.

Remove the clip G and paper from the arm D. Move C until the indicator A is at zero on the scale. See that the lever E is in such a position that F points to "Free." The movable arm B should now be at O. If this is not so, bring B to O by means of an adjusting screw on the back of the instrument.

Now set F to "Stop" by means of E. Hang the clip and paper on to D, seeing that the paper hangs freely. By means of C move the lever A to mark about 120 mgm., set F to "Free," and then move C until B is at O. The reading at A is the weight of the paper and the clip. After the blood has been drawn on to the paper, the weight is again taken as before. This should be done as rapidly as possible to avoid errors due to evaporation.

The paper and clip should never be put on or taken off D with F at "Free." The indicator should be set at the approximate weight before the spring is released. By taking these precautions the instrument will remain reliable for a very long time.

## THE PREPARATION OF CERTAIN REAGENTS AND LABORATORY REQUISITES.

*Acid potassium phosphate*, see p. 24.

*Acid potassium phthalate*, see p. 24.

*Almén's reagent*. 4 grams. of tannic acid in 8 cc. of strong (33 per cent.) acetic acid and 190 cc. of 50 per cent. alcohol.

*Alpha-naphthol*. 1 per cent. in strong alcohol.

*Ammonium molybdate*. Rub up 75 grams. of crystalline ammonium molybdate with 300 cc. of strong ammonia. When it has dissolved gradually add the solution to a mixture of 900 cc. of concentrated nitric acid (sp. gr. 1.42) and 400 cc. of distilled water, cooling thoroughly during the addition. Add 1600 cc. of distilled water and filter, if necessary.

*Ammonium oxalate*, 0.2 N. 1.42 per cent. of  $(\text{COO.NH}_4)_2\text{H}_2\text{O}$ .

*Ammonium sulphate*, saturated solution. Boil 800 grams. of pure crystalline  $(\text{NH}_4)_2\text{SO}_4$  with about 1 litre of distilled water. Filter when cold.

*Ammonium sulphide* is usually purchased. Can be prepared by saturating ammonia (1 part of concentrated to 2 parts of water) with sulphuretted hydrogen and then adding to this one-third of its volume of ammonia of the same dilution.

*Asbestos pulp* for Gooch crucibles, etc. Cut some long-fibred, soft asbestos into pieces about a quarter of an inch long and digest with concentrated hydrochloric acid in a large flask in a water bath for an hour, shaking thoroughly at intervals. Filter through a plate or on a Buchner under light suction and wash thoroughly with water. Transfer to a large wide-neck stoppered bottle and shake thoroughly with water. A good quality asbestos forms a sludge, which allows of rapid filtration, provided that it be not unduly compressed by too great a suction.

To prepare a Gooch crucible for gravimetric analysis, set up the apparatus shewn on p. 351. Pour on enough of the asbestos sludge to form a layer 1 to 2 mm. thick. On this place a perforated porcelain plate, the diameter of which is slightly less than that of the crucible, and then add another layer of asbestos. Filter under light pressure and pass water through until the filtrate is absolutely clear. The crucible can then be dried in a hot air oven at 110° C., cooled and weighed. The drying and weighing should be repeated until the weight is constant. It should then be tested by passing about 500 cc. of water through it, drying and weighing. If constant it is ready for use. The same crucible can be used for a large number of determinations.

*Barfoed's reagent*, see p. 108.

*Barium chloride*, N. 122 grams. of  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$  to 1 litre.

*Baryta mixture*. One vol. of barium chloride solution is added two vols. of baryta water.

*Baryta water*. One part of crystalline barium hydroxide is dissolved in 15 parts of boiling water and filtered hot. The filtrate on cooling throws down crystals of barium hydroxide. The supernatant fluid is baryta water. It is about 0.25 Normal.

*Benedict's solution*, qualitative, see p. 107.

*Benedict's solution*, quantitative, see p. 127.

*Benzidine hydrochloride*, see p. 357.

*Bromine water*, saturated. Made by shaking bromine with cold distilled water.

*Brücke's reagent*, see p. 37.

*Calcium chloride*, normal. 55.5 grams. pure anhydrous  $\text{CaCl}_2$ , dissolved in water, made up to 1 litre and filtered. One-fifth normal is a convenient strength for certain exercises.

*Charcoal, adsorbent*. The author has found that certain samples of the charcoal prepared by the Chemical Warfare Department for filling protective gas masks are highly efficient, being superior to Merck's "blood charcoal." It is hoped that carefully selected supplies will shortly be obtainable from Messrs. Baird and Tatlock's and other dealers.

*Chromic acid cleaning fluid*. Ten per cent. of chromic acid in water, or 10 per cent. of potassium bichromate dissolved in 10 per cent. (by volume) of sulphuric acid.

*Cochineal tincture*, see p. 357.

*Collodion solution*, see p. 3.

*Congo red paper*. White filter paper is thoroughly wetted with a 0.2 per cent. solution of congo red in water. The paper is pinned up till dry and cut into strips. It is turned blue by strong acids.

*Copper sulphate*. 200 grams. of pure crystalline  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  are dissolved in distilled water by the aid of heat, cooled and made up to 1 litre. For the biuret reaction a 1 per cent. solution is prepared by diluting 5 cc. to 100 cc.

*Ehrlich's reagent for indol*. Para-dimethyl-amido-benzaldehyde 4 parts  
Alcohol (95 to 98 per cent.) .. 380 "  
Concentrated hydrochloric acid 80 "

*Esbach's reagent*, see p. 36.

*Fehling's solution*, see p. 106.

*Ferric chloride*, 10 per cent.

*Glyoxylic reagent*, see p. 39.

*Grease paint*, for marking beakers, etc. Dilute Brunswick Black to the desired consistency with naphtha or benzene. Apply with a fine brush. Can be scraped off or removed by means of a pad of cotton wool soaked in naphtha.

*Gunsberg's reagent*. Dissolve 2 grams. phloroglucin and 1 gram. of vanillin in 30 cc. of absolute alcohol. The solution should be freshly prepared, but it can be preserved for a certain time in dark bottles.

The most economical way of preparing it is to make 10 per cent. solutions of phloroglucin and of vanillin in absolute alcohol. These keep for a long time when not mixed. When wanted the reagent is made by taking 1 cc. of the phloroglucin and 0.5 cc. of the vanillin solutions and mixing.

*Iodine solution*. About 0.1 N. Dissolve 25 grams. of potassium iodide in about 200 cc. of distilled water in a stoppered flask. Add 12.7 grams. of iodine and shake till dissolved. Make up to 1 litre with distilled water. For many purposes this can be diluted 10 times or even more with distilled water, but these weak solutions should be prepared as required.

*Lead acetate (basic)*. Boil 464 grams. of normal lead acetate and 264 grams. of litharge in 1500 cc. of distilled water for half an hour with constant stirring. Cool and filter. Or use a saturated solution of the commercial basic lead acetate.

*Lead acetate (normal).* Saturated solution.

*Litmus solution.* Extract the crushed litmus several times with warm distilled water, mix the extracts and filter. Adjust the solution to a neutral tint by means of hydrochloric acid. The sensitiveness of the indicator is much increased by dialysing it against distilled water. A drop or two of chloroform may be added to the solution to prevent the growth of organisms.

*Mercuric chloride.* Saturated solution, about 8 per cent.

*Mercuric nitrate.* A. To 160 cc. of concentrated nitric acid (sp. gr. 1.42) in a beaker add, in small portions, 220 grams. of red mercuric oxide. Stir well and then add 160 cc. of distilled water. Heat till the oxide has dissolved. Cool and nearly neutralise by adding 75 cc. of N. soda. Make up to 1 litre and filter. Preserve in a dark-coloured bottle. This solution is used for removing various nitrogenous substances from urine when estimating small quantities of sugar (see p. 347).

B. To 143 grams. of pure mercury in an evaporating basin add 200 cc. of concentrated nitric acid (sp. gr. 1.42). Heat until thick fumes are evolved and then turn out the gas. When the reaction has ceased light the flame again and evaporate down to about 80 cc. Gradually add about 1500 cc. of water. Cool and make up to 2 litres.

*Mercuric sulphate reagent* for tryptophane. See p. 89.

*Millon's reagent.* See p. 39. It is usually purchased.

*Nessler's solution.* Folin and Denis, *Journ Biol. Chem.*, xxvi., p. 478.

*Phosphotungstic acid.* Two per cent. in 5 per cent. sulphuric acid.

*Picric acid,* saturated solution, about 1.2 per cent.

*Potassium ferricyanide.* Saturated solution, prepared by grinding the solid with cold water in a mortar.

*Potassium ferrocyanide,* 5 per cent.

*Roberts' reagent,* see p. 304.

*Sodium hypobromite.* Dissolve 100 grams. of caustic soda in 250 cc. of water. Cool. **Cautiously** add 25 cc. of bromine, cooling thoroughly at intervals. It must be recently prepared.

*Soluble starch.* 250 grams. of potato starch is placed in a litre flask. It is treated with a mixture of 375 cc. of water and 125 cc. of pure concentrated hydrochloric acid and the mixture thoroughly shaken until the whole of the starch has been wetted by the acid. It is allowed to digest at room temperature for 8 days, being frequently shaken. The acid is then poured off, the residue repeatedly washed with distilled water and then filtered on a Buchner. To remove the last traces of acid, which inhibit the action of enzymes, it is advisable to suspend the starch in a buffer solution of  $P_H = 7$ . This can be prepared approximately by treating 50 cc. of 0.2 M. acid potassium phosphate (see p. 24) with 30 cc. of 0.2 N. soda and diluting to 500 cc. After standing for some time with frequent shakings the starch is again washed by decantation with distilled water, filtered on a Buchner and dried in the air. Solutions are prepared in the manner described for starch paste (p. 120).

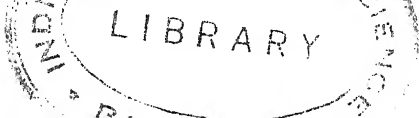
*Stokes' reagent,* see p. 245.

*Sulphosalicylic acid,* see p. 37.

*Tannic acid,* see Almén's reagent.

*Tap grease.* Heat together in a crucible on a sand bath 1 part of soft rubber (free from inorganic filling matter), 1 part of paraffin wax and 2 to 3 parts of vaseline. Stir thoroughly until the rubber has completely dissolved.

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